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THE INFLUENCE OF THE SUBSTRATE CONCENTRATION ON THE RATE OF HYDROLYSIS OF PROTEINS BY PEPSIN.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 18, 1920.)

In contrast to the numerous papers on the influence of changes in the pepsin concentration, the influence of varying the protein concentration on the rate of digestion of protein has been but little studied. Weis[1] found that the rate was nearly directly proportional to the protein concentration in low concentration but increased more slowly than the latter in concentrations of more than 2 to 3 per cent. The experiments were made with a crude enzyme preparation which contained several proteolytic enzymes, and were made in such a way as to compare the changes in different solutions after the same time interval, instead of comparing the times required to cause an equal change. They are therefore difficult to interpret.

Preliminary experiments made with a purified pepsin and purified egg albumin showed in general the same results as those found by Weis. In concentrations of more than 2 to 3 per cent the rate of digestion increases more slowly than the protein concentration and finally becomes nearly independent of it. This phenomenon is a very general one in enzyme reactions and many explanations have been offered to account for it. Brown[2] suggested that the relative decrease in the rate of digestion with increasing substrate concentration was due to the fact that the enzyme remained combined with the substrate for a period of time large compared with the time necessary for combination to take place. The enzyme therefore becomes more and more "saturated" with substrate as the relative concentration of substrate to enzyme increases. Van Slyke and Cullen[3]

[1] Weis, *Med. Carlsberg Lab.*, 1903, v, 127.

[2] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

showed that an equation might be derived on the assumption of the above mechanism, based on the law of mass action. The validity of this derivation has, however, been questioned by Falk.[4] Bayliss[5] has advocated the view that the combination is due to adsorption and cannot be considered a mass action phenomenon. He states, as do Armstrong and Armstrong,[6] that the fact that the rate of hydrolysis in many cases is nearly independent of the total substrate concentration cannot be explained on the law of mass action, and must be due to some saturation effect of the enzyme with the substrate.

The fact is frequently overlooked that purely chemical catalysis in strictly homogeneous solutions also shows apparent divergences from the mass law. This point is, however, discussed by Bredig,[7] Mellor,[8] Lewis,[9] and especially by Falk.[10] In the hydrolysis of cane sugar by acids, for instance, the rate of hydrolysis is not proportional to the total concentration of acid used but to the hydrogen ion concentration. As is well known, the hydrogen ion concentration in heavily "buffered" solutions is almost independent of the total acid concentration in certain ranges so that the hydrolysis of cane sugar by such a solution would show an analogous behavior to enzyme reactions in that the rate of hydrolysis of the sugar would be nearly independent of the total acid concentration. The apparent discrepancy of the mass law is therefore due to the fact that the "active concentration" (on which the mass law is based) is in many cases not identical with the total concentration. There is a further discrepancy in these cases due to the fact that the rate of hydrolysis in certain concentrations is not proportional to the C_H^+ as determined by the conductivity ratio. This is the so called salt effect and is probably

[4] Falk, K. G., *J. Biol. Chem.*, 1916–17, xxviii, 389.

[5] Bayliss, W. M., The nature of enzyme action, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

[6] Armstrong, E. F., and Armstrong, H. F., *Proc. Roy. Soc. London, Series B*, 1913, lxxxvi, 561.

[7] Bredig, G., *Ergebn. Physiol., Ite Abt.*, 1902, i, 134.

[8] Mellor, J. W., Chemical statics and dynamics, London, 1904.

[9] Lewis, W. C. McC., A system of physical chemistry, London, New York, Bombay, Calcutta, and Madras, 1919, i.

[10] Falk, K. G., (in press).

due to increased activity of the hydrogen ions by the salt. In any case, it is not due to any peculiarity of the catalytic reaction since the same discrepancy is found in comparing the hydrogen ion concentration as determined by the conductivity and E.M.F. methods. The apparent discrepancy between the mass action law and the kinetics of acid catalysis as outlined above is analogous to the case in enzyme reactions where the rate is not proportional to the enzyme concentration.

Acid hydrolysis, moreover, also shows the same peculiarity in regard to the sugar concentration, *i.e.* the rate does not increase directly as the sugar concentration, as expressed in grams or molecules per liter. In the case of acid hydrolysis the rate increases more rapidly than the concentration. Arrhenius[11] has suggested that this behavior is due to the fact that the active concentration of sugar is not correctly expressed by the molecular concentration and has shown that very much better results are obtained if the osmotic pressure of the sugar solution is used as a measure of the active concentration. He further assumes that the acid affects the equilibrium between active and inactive sugar molecules and so accounts for the "salt effect." The same mechanism is assumed to account for the effect of temperature, which is much greater than that predicted by the kinetic theory. This hypothesis, of course, fits the facts, but in the absence of independent evidence is really an assumption of the law of mass action rather than a proof of the law. Several authors have proposed explanations for catalytic reactions on the same basis; *i.e.*, that the catalyst changes the concentration of certain molecules and so increases the speed of the reaction. Stieglitz[12] and his coworkers have been able to verify this hypothesis experimentally in the case of the acid hydrolysis of imido esters. This reaction shows the same peculiarities as that found in many enzyme reactions; namely, the rate is not proportional to the total ester concentration. Stieglitz was able to show, however, that the rate was directly proportional to the concentration of ester ions. He considers that the acid causes the formation of imido ester salts and

[11] Arrhenius, S., *Z. physik. Chem.*, 1899, xxviii, 317.

[12] Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 164, 402, 437, 586, 719.

thereby increases the concentration of active ions. He was able to confirm this by independent measurement of the ion concentration (by means of the conductivities). It follows, as emphasized by Stieglitz, that if the above mechanism is correct so called catalytic reactions are merely limiting cases of ordinary reactions in which the combination of the "catalyst" with the substrate or with the products of hydrolysis is too small to be measured. It seems probable that enzyme reactions are of the same type. There is no doubt, at least, that the enzyme often combines with the products of the reaction and so shifts the equilibrium. Bodenstein and Dietz[13] have shown experimentally that this is true in certain cases. It would seem better, therefore, to consider enzyme reactions as cases of bimolecular reaction in which one of the products dissociates more or less completely with the liberation of active enzyme; if the dissociation is complete the result would be a monomolecular reaction and, if no dissociation whatever takes place, a bimolecular reaction. Most enzyme reactions are apparently intermediate. The specificity of enzyme reaction thus becomes neither more nor less remarkable than the specificity of any other chemical reaction. (The author has had the privilege of discussing the above points with Dr. K. G. Falk who has reached independently similar conclusions.) It was shown in a previous paper[14] that the above conception of enzyme reactions as applied to pepsin gives a quantitative explanation for the kinetics of the reaction and explains the fact that the rate is not always proportional to the total concentration of pepsin. Arrhenius[15] has pointed out that it also gives the explanation of Schütz's rule and the divergence from the monomolecular law.

It is clear from the brief account of catalytic reactions given above that the same apparent divergences from the law of mass action are to be found in these reactions as in enzyme reactions and that the divergences in many cases at least are caused by the fact that the active concentration is not the same as the total concentration of substance. It seems quite probable that the same explanation applies to both. There is no doubt that the saturation theory is sufficient

[13] Bodenstein and Dietz, *Z. Elektrochem.*, 1906, xii, 605.

[14] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

[15] Arrhenius, S., *Med. Nobelinst.*, 1908, i.

to explain many of the facts but in the absence of direct evidence it can hardly be considered proved.

According to this theory a certain amount of enzyme can act only on a limited amount of substrate; after this quantity is reached any excess of substrate has no effect on the reaction. It is clear that according to this mechanism it is the ratio of the concentration of substrate to that of the enzyme which causes the relative decrease in the rate of digestion of the substrate as the concentration of substrate increases, and not the actual concentration of substrate present in the solution. If the effect, however, is due to the fact that the active concentration of substrate is not directly proportional to the total concentration then the falling off of the rate of reaction with increasing substrate concentration is independent of the ratio of substrate to enzyme and depends only on the actual concentration of substrate.

It occurred to the writer that this question might be tested experimentally by comparing the rate of digestion of different substrate concentrations when hydrolyzed with different enzyme concentrations. Assume, for instance, that the substrate at concentration 10 S is found to hydrolyze five times as rapidly as the substrate at concentration S, when enzyme concentration E is used. According to the monomolecular formula the substrate at concentration 10 S should digest ten times as rapidly as the substrate at concentration S. The saturation hypothesis would explain this divergence by the assumption that the enzyme becomes saturated with substrate at a concentration of the latter of less than 10 S. In concentration 10 S, therefore, much of the substrate takes no part in the reaction and the rate of reaction is less than the expected. It would be predicted further that increasing the substrate concentration from 10 S to 20 S would have relatively less effect on the rate of reaction than increasing the substrate concentration from S to 2 S. This is true. It follows also on the saturation hypothesis that increasing the enzyme concentration from E to 10 E should have a relatively greater effect on the rate of digestion of substrate 10 S than on the rate of digestion of substrate at concentration S; since it was assumed in accounting for the effect of increasing the substrate concentration that the enzyme (at concentration E) was more saturated with substrate at (substrate) concen-

tration 10 S than at (substrate) concentration S. According to the saturation theory, the rate of digestion in concentration 10 S is limited only by the concentration of enzyme while the rate at concentration S is limited both by the concentration of enzyme and by the concentration of substrate; hence changing the enzyme concentration should have a greater effect at substrate concentration 10 S than at substrate concentration S. The experiments show that this prediction is not fulfilled. The relative increase in the rate of digestion of substrate at concentration S, caused by increasing the concentration of enzyme from E to 10 E, is identical with the relative increase in rate of digestion of the substrate at concentration 10 S, caused by the same increase in enzyme concentration.

If, on the other hand, the relative decrease in rate with increase in concentration of substrate is due to an equilibrium in the substrate solution which causes the concentration of active molecules to differ from the total concentration, the rate of hydrolysis of the substrate at concentration 10 S should be always five times the rate of digestion at concentration S (in the example just discussed), irrespective of the enzyme concentration. Experiments show that this is actually the case. It is necessary, of course, in making such experiments to be sure that the range covered is such that the enzyme cannot be considered saturated in both substrate concentrations. That is, the range of substrate concentrations must be such as to show nearly direct proportionality between the rate of digestion and the substrate concentration in the lower, but not in the higher concentrations of substrate. It is also necessary to measure the time required to cause a constant change in the substrate and not a constant percentage change or the change made in a given time. The failure to recognize this has led to much confusion in discussion of the kinetics of enzyme reactions (*cf.* Bredig).[7]

This is due to the fact that in most enzyme reactions the products retard the action of the enzyme. It will be clear therefore (irrespective of the mechanism by which this retardation takes place), that comparative results can be obtained only when a constant amount of products is formed. The actual amount of products formed for example by 10 per cent hydrolysis of varying substrate concentrations will be very different. The larger the concentration of substrate the greater the amount of products formed by 10 per cent hydrolysis and the greater

the consequent slowing up of the enzyme due to the inhibiting effect of the products. It is also clear that the retardation will be proportionally greater if a small amount of enzyme is present than if a large amount is present (irrespective of the mechanism by which the retardation is affected). The same reasoning holds for the case when the amount of products formed in a given time is taken as the measure of the rate of reaction. This question was discussed fully in a previous paper.[14]

In all the experiments given in this paper, therefore, the rate of digestion is measured as the reciprocal of the time necessary to cause a small absolute change in the substrate concentration. According to the law of mass action as applied to monomolecular reactions the time necessary to cause this change should be nearly inversely proportional to the substrate concentration, provided the change is small compared to the total change in the lowest concentration. If wider variations than this are used it is necessary to calculate the predicted time according to the monomolecular formula. It may appear that the above method of testing the reaction is a very indirect one and that a simpler and more exact method would be to express the course of a single reaction, according to the mechanism proposed, in a single equation. This equation could then be tested experimentally. Such a procedure, however, leads inevitably to an equation with two or more constants, the value of which must be determined from the experiments themselves, so that but little weight can be attached to the agreement of such an equation with the experimental facts. It seems better, therefore, to limit the experimental conditions in such a way as to leave but one variable.

In all the experiments reported in this paper, the changes are within the above limits and the time required to cause a constant change should, therefore, (according to the mass law) be nearly inversely proportional to the substrate concentration at the beginning of the reaction. As will be seen this is not the case if the total concentration of protein is considered as the active concentration but is approximately true if the concentration of ionized protein is considered as the reacting mass.

In these experiments the rate of hydrolysis was followed by means of changes in the conductivity of the solution. It is, therefore, necessary to be sure that the production of the same amount of peptone in each of the solutions used causes the same change in conductivity. This was tested experimentally in each experiment by adding 1 cc. of peptone solution (prepared from egg albumin by the action of pepsin) to 25 cc. of the protein solution and determining the change in conductivity. It was found that the addition of an equal amount of peptone to protein solutions of varying concentrations (from 20 to 1 per cent) does cause an equal change in conductivity provided the hydrogen ion concentration of the solution is greater than pH 1.8. If the solution is less acid than this the change in conductivity of the solution on the addition of a constant quantity of peptone in the presence of a large amount of protein is less than that caused in the presence of a small amount of protein. This is obviously due to the buffer action of the protein in high concentration and can be foreseen from the titration curve of the protein.

Fig. 1 gives the results of two experiments on the effect of the pepsin concentration on the relative rate of digestion of protein solutions of different concentration. In Experiment 1, (Curve I, Fig. 1) 25 cc. of protein solution containing 8, 4, 2, 1, and 0.5 per cent protein, were hydrolyzed at 25° with the addition of (*a*) 1 cc. of 2 per cent pepsin, and (*b*) 1 cc. of 0.2 per cent pepsin. All solutions were brought to a pH of 1.8 with hydrochloric acid. The time necessary to cause a given change (about 1.4×10^{-4} reciprocal ohms) in the specific conductivity was determined.[16] The reciprocal of this

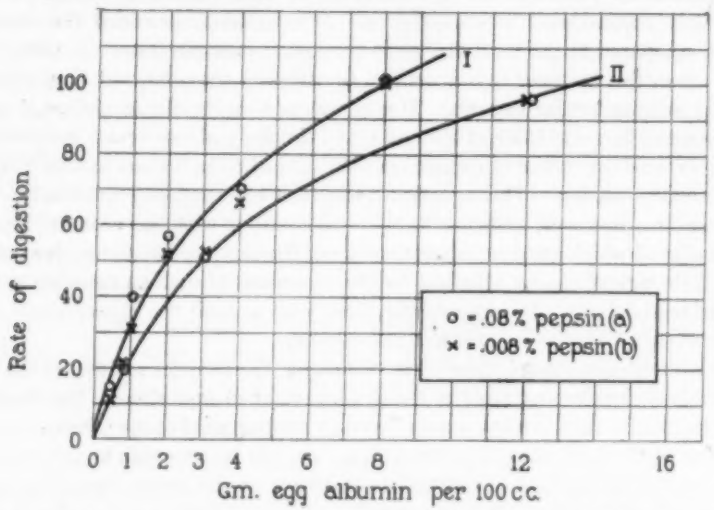

FIG. 1. Relative rate of digestion of egg albumin solutions of different concentration when digested with pepsin solutions of different concentration.

time, therefore, gives the mean rate of digestion of the various solutions for the first 1.4×10^{-4} reciprocal ohm change. In order to compare the two series, the rate of digestion of the concentrated egg albumin (in each series) was considered as 100 and the rate of digestion of the other concentrations calculated on this basis. The curve shows that the relative rate of digestion of the 8 per cent egg albumin compared to the rate of digestion of 4, 2, 1, or 0.5 per cent egg albumin is the same irrespective of whether 2 or 0.2 per cent pepsin was used. The curve also shows that in low concentrations, 0.5 to 2 per cent, the increase in rate is nearly proportional to the increase

[16] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

in substrate concentration but increases much more slowly in high concentrations. Experiment 2, (Curve II) shows the same result. It was made the same way but at a pH of 1.6. The points in this case are the average of two determinations. They are therefore more reliable and as the figure shows also more nearly equal. In both experiments the relative activities of the two pepsin solutions were as 4.7: 1. It follows from the experiments that this ratio is also inde-

TABLE I.

Influence of Pepsin Concentration on Relative Rate of Digestion of Protein Solutions.

Ratio: $\frac{\text{Rate of hydrolysis of 15 per cent albumin}}{\text{Rate of hydrolysis of 1 per cent albumin}}$ with	
0.08 per cent pepsin.	0.008 per cent pepsin.
8.9	9.1
9.4	9.4
10.0	10.0
9.8	9.7
Average..... 9.52	9.54
Ratio: $\frac{\text{Rate of hydrolysis with 0.08 per cent pepsin}}{\text{Rate of hydrolysis with 0.008 per cent pepsin}}$ in	
15 per cent protein.	1 per cent protein.
5.3	5.2
5.0	5.4
5.0	5.0
4.7	5.6
5.3	5.5
Average..... 5.06	5.34

pendent of the substrate concentration in which the tests were made. (It was shown in a previous paper[14] that the discrepancy in the rate of digestion as compared with the enzyme concentration can be quantitatively explained on the basis of a mass action equilibrium between the pepsin and peptone.) Table I shows a similar experiment in which several duplicate determinations were made at two protein concentrations with two enzyme concentrations. The results are more accurate and also in closer agreement than those shown in Fig. 1.

It seems necessary to conclude from these experiments that the relative decrease in the rate of digestion of protein solutions of increasing concentration is independent (within the limits of error of these experiments) of the enzyme concentration used.

There does not appear to be any direct experimental evidence on the above point in connection with other enzymes. It is frequently stated, however, (Nelson and Vosburgh,[17] Van Slyke and Cullen[3]) that the velocity of reaction is directly proportional to the enzyme concentration under all conditions and irrespective of the substrate concentration. If this is true it follows necessarily that the relative rate of digestion of various substrate concentrations, when hydrolyzed with any given enzyme concentration, is independent of the enzyme concentration used.

It appears to the writer that this is contrary to the result predicted by the saturation theory. According to this theory it would be predicted that the falling off in the increase in the rate of digestion as compared to the increase in concentration of a protein solution (above a certain low concentration) is due to the fact that at this concentration the enzyme begins to become saturated with substrate; *i.e.*, the time necessary for the enzyme to combine with the substrate becomes small compared with the time during which it remains combined. If this saturation effect becomes noticeable at a concentration of protein of 2 per cent with 0.08 per cent pepsin it should become noticeable at a lower protein concentration with 0.008 per cent pepsin. The experiment shows this is not the case. If anything, the figures show that the rate of digestion of the substrate falls off more rapidly (as compared to the concentration) with the higher pepsin concentration than with the lower. In Table I, which is more reliable owing to the larger number of determinations there is less than 1 per cent difference.[18]

It seems necessary to conclude therefore that the relative decrease in the rate of digestion as compared with the increase in protein con-

[17] Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790.

[18] It probably cannot be assumed that, according to the saturation theory, the rate of digestion (caused by increasing the enzyme concentration ten times) should be increased ten times as much in the concentrated as in the dilute substrate concentration. The increase should be large enough to detect, however.

centration is due to some equilibrium in the protein solution itself and is independent of the enzyme concentration.

It is well known that in acid solution protein exists in an ionized condition. The concentration of ionized protein is not directly proportional to the total concentration but will increase more slowly than the total concentration. It is obvious, therefore, that the rate of digestion will be more nearly proportional to the concentration of ionized protein than to the total concentration of protein. The hypothesis, then, that the ionized protein is the form which takes part in the reaction, will allow a nearer approach to the predicted rate of reaction. Pauli[19] has suggested that the enzyme attacks the ionized protein; there seems, however, to be no direct evidence for this view. It can be tested experimentally by comparing the rate of digestion with the degree of ionization of the protein.

The concentration of ionized protein can be determined approximately from the pH and conductivity measurements. If the total conductivity and the hydrogen ion concentration of a solution are accurately known, the conductivity due to the protein-salt ions can be determined by subtracting the conductivity of the free HCl from that of the solution. The validity of this method rests on three conditions: (1) the conductivity of the free HCl in the solution is the same as that of the same concentration of acid in water solution; (2) the C_{Cl^-} is equal to or greater than the C_H^+; and (3) the hydrogen ion concentration as determined by the E.M.F. method must agree with that found by the conductivity method.[20] The first assumption cannot be tested directly but it has been shown by Hardy[21] and by Loeb[22] that the viscosity of the solution has no significant effect on the conductivity since the viscosity may increase till the solution is nearly solid without an appreciable change in the conductivity. This experiment was repeated and confirmed. The second condition can be shown to hold also by direct measurements of the chlorine ion concentration by means of concentration cells as was done by Manabe and Matula.[23] Many measurements of this kind were made and confirmed those of the above mentioned authors; namely, the chlorine ion concentration is always equal to or greater than the hydrogen ion concentration. It was

[19] Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483.

[20] For the purpose of these experiments it is only necessary that the conductivity and E. M. F. methods should agree. The absolute value for the C_H^+ is immaterial.

[21] Hardy, W. B., *J. Physiol.*, 1905, xxxiii, 251.

[22] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

[23] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

found, however, that if the conductivity of the H^+ and the total Cl^- were subtracted from that of the solution the remaining values were within the limits of error of the measurements. In other words, the conductivity due to the protein ion itself is very small compared to that due to the excess chlorine ion (by excess Cl^- is meant the difference between the total C_{Cl-} and the $C_{Cl-} = C_H^+$). Since, however, the protein ion must equal in concentration the excess chlorine ion the value for the conductivity obtained by subtracting the conductivity of the free HCl from that of the solution may be considered as proportional to the amount of ionized protein. (Recent work, by Noyes, Milner, and others, has rendered questionable the exact interpretation to be put upon conductivity ratios; they very probably do not represent the actual ion concentration in all cases.) The third condition may be experimentally fulfilled by standardizing the apparatus used for the C_H^+ determinations against HCl solutions of known conductivity and taking the C_H^+ as that determined by the conductivity ratios. This method was used in the present experiments. The final values for the conductivity due to the protein-salt ions are the difference between two large figures so that the error is very large and becomes larger as the solution becomes more dilute. Below 1 per cent protein solution (at pH 1.7) the value is meaningless as it usually lies within the limit of error.

The egg albumin was crystallized three times and then dialyzed under pressure at the isoelectric point until the specific conductivity was lower than 1×10^{-4} reciprocal ohm. The solutions were then brought to a pH of 1.6 to 1.8 with HCl and then diluted with HCl of exactly the same pH. The solutions varied from 16 to 1 per cent egg albumin. The time necessary to cause a constant small change in the conductivity of the resulting solution by the same amount of pepsin was then determined as described previously.[16] The reciprocal of this time is plotted in the curves as the rate. The conductivity of the solution was measured on an aliquot part of the solution to which the equivalent amount of inactivated pepsin had been added. The C_H^+ was determined by the E.M.F. method on this solution. The value given for the specific conductivity of the protein is obtained by subtracting the specific conductivity of the free HCl from that of the solution. The experimental error of the value is 5 to 10 per cent in the high concentrations and 20 to 30 per cent in the lower. The figures given are the averages of three determinations. All measurements were made at 25° ± 0.01.

The conductivity and rate of digestion of the egg albumin was measured in this way. It was found in general that the conductivity of the protein solution was, within the rather large limits of error, directly proportional to the rate of digestion of the solution. In other words, the rate of digestion is that predicted by the mass law if the ionized protein is considered as the reacting form. The results of three such experiments are given graphically in Fig. 2 in which

the rate of digestion is plotted against the conductivity of the protein. This figure shows that the two values are approximately directly proportional.

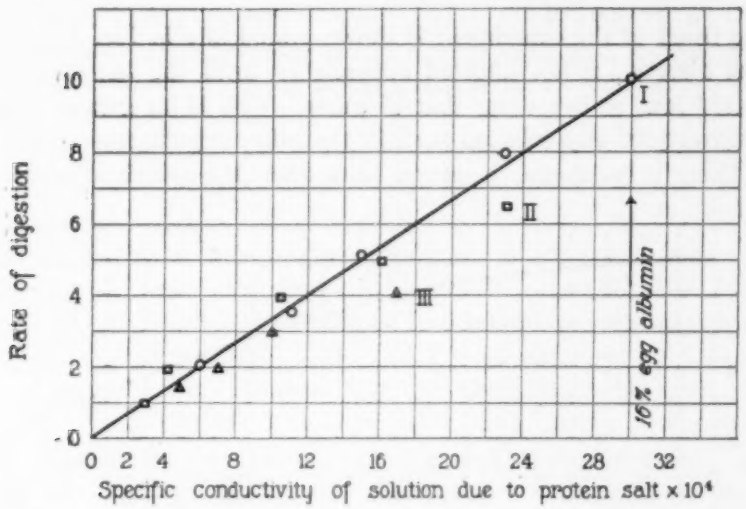

FIG. 2. Rate of digestion and conductivity of egg albumin solutions.

Viscosity of the Solution.

It is impossible to use egg albumin in more concentrated solutions than 16 per cent as the higher concentrations set to a jelly very rapidly. It seemed quite possible that the increasing viscosity of the solution might affect the rate of digestion (as found by Colin and Chaudun[24] for invertase). This question can be tested experimentally by taking advantage of the well known hysteresis of albumin solutions.

500 cc. of a 25 per cent egg albumin solution were titrated to pH 1.6 with HCl, placed at 25°, and the viscosity and rate of digestion of a sample determined at intervals for about 10 hours. The amount of pepsin used was such that the viscosity of the digesting solution did not change appreciably during the determination. This was due to the fact that the decrease in viscosity by the pepsin was equallized by the increase of the viscosity with time. The viscosity of the solution at the beginning of the experiment was about three times that of water and at the end too large to measure by the viscosimeter. At the beginning of the last

[24] Colin, H., and Chaudun, A., *Compt. rend. Acad.*, 1919, clxix, 849.

digestion test the solution could hardly be pipetted with a wide-mouth pipette and was semisolid. The figures for viscosity are doubtless all too low since the viscosimeter was not known to obey Poiseuille's[25] law and almost certainly did not obey it since the time of outflow for 20 cc. of water was only 10 seconds.

The result of the experiment is shown graphically in Fig. 3. The rate of digestion is not affected appreciably, until the viscosity has increased four to five times that of water. This is far greater than the viscosity of any solution used in the other experiments referred to. There is no doubt, however, that when the solution becomes

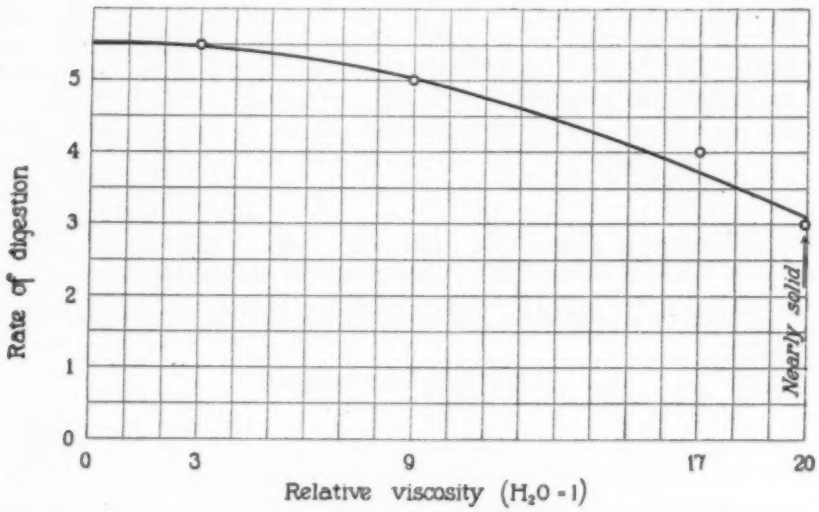

FIG. 3. Influence of viscosity on rate of digestion.

nearly solid the rate of digestion is greatly decreased. It was found that the same decrease was noticed if the viscosity of the solution was increased by the addition of agar. The presence of the agar alone is not the cause of the decrease in the rate as was shown by the fact that the rate of digestion was unaffected by the agar if the experiment was conducted at 40° (when the agar caused no increase in viscosity). It would seem, therefore, that the effect of viscosity must be a mechanical one due to interference with the diffusion of

[25] For a discussion of this question see Washburn, E. W., and Williams, G. Y., *J. Am. Chem. Soc.*, 1913, xxxv, 737.

the enzyme rather than to a change in the resistance of the protein. This is borne out by the fact that pepsin diffuses only very slowly through coagulated protein (*cf.* for instance Dauwe).[26] Reformatsky[27] has shown that the rate of hydrolysis of methyl acetate by acids is identical in water solution and in a solid agar gel. In this case the rate of diffusion of the H^+ is also independent of the viscosity (Voigtländer).[28]

Ringer[29] has pointed out that the optimum pH for the digestion of protein coincides approximately with the maximum viscosity and has suggested that the rate of digestion is dependent on the degree of hydration of the protein; the viscosity of the solution is also assumed to be a measure of the degree of hydration. It would seem from the experiment just described that an increase in viscosity decreases the rate of digestion instead of increasing it, as supposed by Ringer. If the protein ion is the active form of the protein the optimum pH should depend on the maximum degree of ionization. According to Pauli[19] the maximum viscosity also depends on the ionization. Loeb[22] has shown, however, that this is not true. The hypothesis outlined above requires that the rate of digestion of a protein solution at different pH should be directly proportional to the amount of protein ionized. Preliminary experiments show that this is true, qualitatively at least. Unfortunately the change in conductivity (as pointed out above) cannot be used to follow the rate of digestion at lower C_H^+ so that the experimental difficulties are much greater.

The results of the present paper may be considered in qualitative agreement at least with the mechanism of pepsin digestion as outlined in the preceding paper.[14] The hypothesis advanced considers that there is an equilibrium in the pepsin solution between pepsin and peptone (substances combining with pepsin and so rendering it inactive). There is also an equilibrium between ionized and unionized protein in the protein solution. The reaction takes place according

[26] Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426.

[27] Reformatsky, S., *Z. physik. Chem.*, 1891, vii, 34.

[28] Voigtländer, F., *Z. physik. Chem.*, 1889, iii, 316.

[29] Ringer, W. E., *Arch. Neerl. Phys.*, 1918, ii, 571; *Z. physiol. Chem.*, 1915, xcv, 195. Ringer considers that the charge on the protein is also of importance. This agrees with the present experiments.

to the law of mass action between the uncombined pepsin and the protein ion. The mechanism may be formulated as below.

$$\text{Pepsin} + \text{peptone} \leftrightharpoons \text{pepsin-peptone}$$

or

$$C_{\text{pepsin}} = K \frac{C_{\text{pepsin-peptone}}}{C_{\text{peptone}}} \qquad (1)^{30}$$

and

$$\text{Protein-chloride} \leftrightharpoons \text{protein}^{+} + Cl^{-}$$

or

$$C_{\text{protein ion}} = K \frac{C_{\text{protein-chloride}}}{C_{Cl^-}} \qquad (2)^{30}$$

The reaction would be expressed by

$$\text{Protein ion} + \text{pepsin} \rightleftharpoons [\text{protein ion-pepsin}] \rightleftharpoons \text{peptone-pepsin} \rightleftharpoons \text{pepsin} + \text{peptone}$$

The rate of hydrolysis of the protein at any instant of time would therefore be proportional to the concentration of protein ions and of free pepsin present in the solution at that instant and the differential expression for the rate of reaction would be

$$-\frac{dC_{\text{protein ion}}}{dt} = K\, C_{\text{protein ion}} \cdot C_{\text{pepsin}}$$

where $C_{\text{protein ion}}$ and C_{pepsin} are determined by equations (1) and (2). There is probably little doubt that the enzyme and substrate unite to form an addition product, but according to the experimental evidence found in this paper the time during which they are combined is negligible in the consideration of the kinetics of the reaction.

The mechanism outlined above will explain, at least qualitatively, the peculiarities in the kinetics of other enzyme reactions. It seems very unlikely, however, that the equilibrium in the substrate solution should always be ionic. It may be any isomeric equilibrium. Since,

[30] The equilibrium expressed in (2) is certainly, and that expressed in (1) is probably, influenced by the hydrogen ion concentration.

in such cases, it is extremely difficult to obtain any independent measurement of the equilibrium, there seems to be no way to test the proposed mechanism.

SUMMARY.

1. It is pointed out that the apparent exceptions to the law of mass action found in enzyme reactions may be found in catalytic reactions in strictly homogeneous solutions.

2. These deviations in the rate of reaction from the law of mass action may be explained by the hypothesis that the active mass of the reacting substances is not directly proportional to the total concentration of substance taken.

3. In support of this suggestion it is shown that for any given concentration of pepsin the relative rate of digestion of concentrated and of dilute protein solutions is always the same. If the rate of digestion depended on the saturation of the surface of the enzyme by substrate the relative rate of digestion of concentrated protein solutions should increase more rapidly with the concentration of enzyme than that of dilute solutions. This was found not to be true, even when the enzyme could not be considered saturated in the dilute protein solutions.

4. The rate of digestion and the conductivity of egg albumin solutions of different concentration were found to be approximately proportional at the same pH. This agrees with the hypothesis first expressed by Pauli that the ionized protein is largely or entirely the form which is attacked by the enzyme.

5. The rate of digestion is diminished by a very large increase in the viscosity of the protein solution. This effect is probably a mechanical one due to the retardation of the diffusion of the enzyme.

ENZYME ACTION IN ECHINODONTIUM TINCTORIUM ELLIS AND EVERHART.

By HENRY SCHMITZ.

(From the Laboratory of Forest Pathology, School of Forestry, University of Idaho, Moscow.)

(Received for publication, March 25, 1920.)

In a recent paper[1] it was pointed out that information concerning the physiology of the wood-destroying fungi is comparatively meager. The intention to investigate the enzyme action in some of these forms was also expressed there. The present paper is the second of a series dealing with this phase of metabolism of the wood-destroying fungi.

Echinodontium tinctorium is perhaps one of the most destructive heart rot diseases in the West. White, Alpine, grand, noble, and Douglas fir, Engelmann's spruce, and western and mountain hemlock have been reported as having been affected. Perhaps by far the greatest damage occurs on white fir and western hemlock. The economic importance of this fungus has been sufficiently discussed by Weir and Hubert[2] and also by Meinecke[3] so that no further discussion of its economic importance or distribution is necessary.

The culture of the fungus used in this study was obtained from a young sporophore by the tissue method. The sporophore was carefully washed with sterile distilled water, dried by means of sterile tissue towelling, and cut open. Small portions of tissue were taken from the interior of the fruiting body and transferred to potato agar slants. After the fungus had made considerable growth, transfers were made from the agar slants to sliced sterile carrots in large Erlen-

[1] Schmitz, H., and Zeller, S. M., Studies in the physiology of the fungi IX. Enzyme action in *Armillaria mellea* Vahl., *Daedalea confragosa* (Bolt.) Fr., and *Polyporus lucidus* (Leys.) Fr., *Ann. Missouri Bot. Garden*, 1919, vi, 193–200.

[2] Weir, J. R., and Hubert, E. E., Forest disease surveys, *U. S. Dept. Agric., Bull. 658*, 1918, 1–23.

[3] Meinecke, E. P., Forest trees common in California and Nevada, *U. S. Dept. Agric., Forest Service*, 1914, 1–67.

meyer flasks, and the cultures incubated for 3 months at a temperature of 32°C. The fungus makes comparatively slow growth both on hard potato agar and on the carrots. While still in an actively growing condition the fungous mats were removed from the flasks, and, when thoroughly dry, were finely ground.

All the methods followed in the present study are similar to those in the former paper.[1] This was done in order to make the results as comparable as possible.

Esterases.

The esterase activity of *Echinodontium tinctorium* was determined by the action of the ground fungous meal on methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion. After 21 days incubation hydrogen ion concentration determinations of the various enzyme cultures and controls were made and compared. Marked esterase activity occurred when methyl acetate and ethyl acetate were used as substrates, a trace of activity when methyl butyrate was employed, but no apparent activity when triacetin and olive oil emulsion were used as substrates.

Carbohydrases.

Carbohydrase activity is, no doubt, the most important and most interesting phase in the study of the physiology of the wood-destroying fungi with reference to enzyme action. The action of the fungous meal was determined on 1 per cent solutions of maltose, lactose, sucrose, raffinose, potato starch, inulin, white fir, filter paper cellulose, and hemicellulose. After varying periods of incubation the enzyme cultures were filtered and 5 cc. samples of the filtrate treated with 20 cc. of Fehling's solution. In Table I the average results of two titrations are given as the number of cc. of 0.05 N potassium permanganate required to oxidize the dissolved copper oxide. The results indicate evident action on all the substrates.

TABLE I.

The Carbohydrase Action of Echinodontium tinctorium.

Incubation period.	Substrate.	With fungous meal.	With fungous meal autoclaved.	Without fungous meal.
		0.05 N $KMnO_4$.		
		cc.	cc.	cc.
14 days	Maltose	31.3	19.1	15.1
20 "	Lactose	27.5	24.7	21.2
3 hrs.	Sucrose	8.2	5.3	0.2
3 days	Raffinose	6.1	4.0	0.3
6 hrs.	Potato starch	4.4	3.1	0.2
6 days	Inulin	7.0	4.5	0.3
30 "	White fir cellulose	8.9	3.6	0.2
25 "	Filter paper "	7.1	3.2	0.4
30 "	Hemicellulose	8.5	4.8	1.5

Tannase.

Tannase activity was determined by the action of the fungous meal on a 1 per cent solution of tannic acid. After 20 days incubation the cultures were filtered and 5 cc. of the filtrates were titrated against 0.05 N iodine. In all cases negative results were obtained.

Amidase and Urease.

Acetamide and urea were used as substrates to determine the presence or absence of enzymes which split amino-acids into ammonia and hydroxyl acids. The enzyme cultures were set up in wash bottles with the intakes and outlets sealed by means of rubber tubing and clamps. After 10 days incubation the bottles were connected up with other wash bottles containing distilled water and a few drops of bromothymol blue; air was then drawn through the series by means of a suction pump. The change in color due to the shifting of the hydrogen ion concentration and the length of time necessary to cause this change were noted.

When acetamide was used as a substrate, all tests were negative. With urea as a substrate, however, the color of the indicator changed from yellowish brown (pH 5.6) to bright blue in 3 seconds. In the

tests in the controls a slight change in color was also noted, but in no case going beyond the light blue stage even after air had been drawn through the wash bottles for 3 minutes.

Rennet.

The presence of rennet has been variously reported in the wood-destroying fungi. In *Echinodontium tinctorium* fresh milk was coagulated in 2 hours while the controls remained unchanged.

Catalase.

When 50 cc. of a 3 per cent solution of hydrogen peroxide were added to 1 gm. of fungous meal, a rapid evolution of oxygen resulted amounting to 42 cc. of the gas in 1 minute.

Proteases.

Tryptic and ereptic fermentations were studied by the use of 1 per cent solutions of albumin, peptone, casein, and fibrin in enzyme cultures having neutral, acid, and alkaline reactions. In no case was a positive result obtained with either the biuret or the tryptophane test. In order to check the negative results in the fibrin, this material was stained with Congo red and the color fixed by immersing in boiling water. This stained fibrin was then used as a substrate. In such cultures a liberation of the stain would indicate the digestion of the fibrin. In the enzyme cultures having an alkaline reaction, a slight liberation of the stain was noted, but this also occurred in the alkaline controls. Biuret and tryptophane tests with these cultures also were negative.

SUMMARY.

In *Echinodontium tinctorium* the presence of the following enzymes was demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, urease, rennet, and catalase.

COMPARATIVE STUDIES ON RESPIRATION.

XI. The Effect of Hydrogen Ion Concentration on the Respiration of Penicillium chrysogenum.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, May 28, 1920.)

The effect of hydrogen ion concentration on respiration has received very little attention. Warburg[1] measured the amount of oxygen consumed by fertilized eggs of *Strongylocentrotus lividus*, when they were in a balanced solution of NaCl, KCl, and $CaCl_2$. He found that on raising the hydrogen ion concentration from pH 8 (which is that of sea water) to pH 6 the oxygen consumption was lowered to nearly one-third, while on lowering the hydrogen ion concentration to pH 11 the oxygen consumption was more than doubled. Loeb and Wasteneys[2] repeated Warburg's experiments, using *Arbacia punctulata*. They found that if oxygen consumption at an OH ion concentration of 10^{-7} (pH 7) is taken as 1.00, at a concentration of 10^{-4} (pH 10) it was 1.17 and at a concentration of 8.4×10^{-4} (pH 10.92) it was 2.74. Since similar concentrations of NaOH and NH_4OH produced similar effects it appeared doubtful whether the result was to be attributed to the concentration of hydroxyl ions only.

Thunberg,[3] studying the effect of H ions on surviving frog muscles, found that with concentrations of 0.005 M HCl (about pH 2.3) the production of CO_2 decreased to 82.7 per cent of the normal, 0.02 M HCl (about pH 1.7) decreased it to 44.6 per cent while with 0.05 M HCl (about pH 1.2) there was a decrease to 24 per cent of the normal. He found the same concentrations of NaOH less toxic; 0.005

[1] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

[2] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvii, 410.

[3] Thunberg, T., *Skand. Arch. Physiol.*, 1910, xxiii, 154; 1911, xxiv, 23.

M (about pH 11.7) decreased the production of CO_2 to 88.8 per cent, 0.02 M (about pH 12.3) decreased it to 71.3 per cent. He found $Ca(OH)_2$ more toxic than HCl at equal concentrations. He explains this by supposing that calcium precipitates out phosphates in the tissue. Oxygen consumption was decreased to about the same degree that the production of CO_2 was decreased. Thunberg also used a large number of organic acids and found that most of them had only a slight effect in concentrations from 0.01 to 0.2 M. A few, like formic, α-oxybutyric, and pyroracemic acids, decreased the production of CO_2 from 40 per cent to 50 per cent at the greatest concentration. On the other hand acids like citric, fumaric, and malic increased the production of CO_2 in proportion to the increase in concentration of acid.

Some work has been done on the effect of H ions on the fermentation by yeast. In most cases, however, the pH of the solutions was not measured. Solutions of 0.1 N HCl and HNO_3 seem to inhibit fermentation entirely.[4] Hägglund[5] determined the pH of his solutions and found that the optimum hydrogen ion concentration for yeast fermentation (using lactic acid, HCl, and H_2SO_4) was 7×10^{-4} (pH 3.16). His criterion of fermentation was the production of CO_2. Lüers[6] found that the pH of the solution, after fermentation had been in progress for several days, always was from 2.51 to 2.73, though at the beginning it may have been neutral.

The extent to which oxidase enzymes are concerned in respiration is at present unknown, but it is important to note that they are inhibited by acids. Bertrand,[7] as well as Abderhalden and Guggenheim,[8] and later Wolff[9] reported inhibition of oxidase reaction by acids. According to Bunzell[10] the oxidation of hydrochinone by laccase (from alfalfa) increases with decrease of H ions from 5.6×10^{-8} to 7×10^{-8} (pH 7.26 to pH 8.16). Reed[11] found that the

[4] Drabble, E., and Scott, D. G., *Biochem. J.*, 1907, ii, 340.
[5] Hägglund, E., *Biochem. Z.*, 1915, lxix, 190.
[6] Lüers, H., *Z. ges. Brauwesen.*, 1914, xxxvii, 79.
[7] Bertrand, G., *Compt. rend. Acad.*, 1907, cxlv, 340.
[8] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.
[9] Wolff, J., *Compt. rend. Acad.*, 1909, cxlviii, 500.
[10] Bunzell, H. H., *J. Biol. Chem.*, 1915, xx, 697.
[11] Reed, G. B., *J. Biol. Chem.*, 1916, xxvii, 299.

oxidase in potato and apple juice was inhibited, when the solution had had hydrogen ion concentrations of 5.5×10^{-4} (pH 3.26) and 7.4×10^{-4} (pH 3.15) respectively, and that in both cases the optimum is near neutrality. Bunzell[12] found that potato juice oxidase was inhibited by pH 3.55 to pH 3.70, and that the activity increased as the pH number increased up to 7. The oxidase of the tulip tree was inhibited at pH 2.30 to pH 2.80, and increased in activity up to pH 6. He also found that the oxidase of magnolia was inhibited at pH 2.45 to pH 3.05 and increased with increasing pH value up to pH 5.70.

In the experiments to be described *Penicillium chrysogenum* was used. This plant was chosen because under the conditions of these experiments it does not produce sufficient alkali or acid (other than carbonic) to interfere with the results. Another reason which made it very good material to work with was its hardiness (it was originally found growing on strong formalin solutions). *Aspergillus niger*, which has been used by the writer in previous work on respiration,[13] produces a small amount of a non-volatile acid, for which reason it was not well suited to the method employed in the present investigation.

The way in which the fungus was grown, as well as the procedure employed in measuring the production of CO_2, and the method employed in calculating the rate are described in a previous paper by the writer.[13]

The pH of the solution in which the fungus was placed to determine the effect of hydrogen ion concentration was determined by means of indicators, using buffer solutions for comparison. For determining the concentrations around pH 1 to 4 tropeolin OO and methyl red were used, with buffers containing HCl and sodium citrate.[14] For the concentrations at pH 7 to 9 phenolsulfonephthalein and phenolphthalein were employed and borate buffers were used for comparison.

The normal respiration was measured in 0.5 per cent dextrose solution in distilled water. To a similar solution sufficient acid or alkali was added to get the desired hydrogen ion concentration.

[12] Bunzell, H. H., *J. Biol. Chem.*, 1916–17, xxviii, 315.

[13] Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 17.

[14] Sörenson, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

NaOH was employed for the alkaline solutions. For the acid solutions H_3PO_4 and H_2SO_4 were used; to get rid of any volatile acid which might be present the acid was boiled for some time before using.

For preliminary work both H_3PO_4 and H_2SO_4 were employed, but as both of these acids gave practically the same results, when the H ions were present in the same concentration, the use of H_3PO_4 was discontinued. All the results here described were obtained by the use of H_2SO_4.

In the experiments where the pH was less than 7 the apparatus described by Osterhout[15] was employed. For the alkaline solutions this method could not be used, as the CO_2 neutralized the NaOH, and therefore the method described by Haas[16] was employed. The method adopted here was to determine the time required to produce a given amount of CO_2 in normal respiration at pH 7.30 and then change the pH and determine the time required to produce the same amount of CO_2.

This necessitated a determination of the change in pH produced by the same amount of CO_2 when added to neutral or alkaline solutions. This was accomplished in the following manner. Into a glass tube 10 cc. of a solution having a pH of 7.30 were put, and into a second tube 10 cc. of an alkaline solution; both solutions contained indicators. As soon as the tubes were filled they were stoppered and compared with standard buffers to get the exact pH. Into each tube one drop of the same solution of CO_2 in water was simultaneously introduced. The tubes were shaken and comparisons with standard buffers were again made in order to measure the change in pH. This procedure was repeated several times and the average of the changes in each solution taken as the change to be produced by the fungus. If for example the average of these changes was found to be from pH 7.30 to 7.10 in one solution, and from pH 8.92 to 8.68 in the other, standard buffers were made up for these values. Then the normal rate of respiration was determined by finding how long it took the fungus to change the pH from 7.30 to 7.10. Unless the rate remained constant for at least 20 minutes the material was rejected. (It had been found by experiment that after the respira-

[15] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

[16] Haas, A. R., *Science*, 1916, xliv, 105.

tion remained constant for 20 minutes it would keep this rate for hours provided food was supplied.) After a constant rate had been established, the material was transferred to the alkaline solution and the rate again determined by noting the time required to produce an equal amount of CO_2; *i.e.*, to change from pH 8.92 to 8.68.

The pH of the nutrient solution both before the spores were sown and after the mycelium had grown large enough to be used was found to be about 4. In no case was there any noticeable change during the growth. A few cultures were grown in a nutrient solution having a pH of 6 to 7. These cultures showed no difference in their behavior from those grown in a more acid medium. The medium on which the fungus was originally growing had a pH of 6 to 7. In all the experiments there was some change in the pH value of the solution containing the fungus. The average pH during the experiment is therefore taken in all cases as the pH of the experiment.

In the following account the rate of respiration at neutrality is arbitrarily designated as normal.

The preliminary work was started with solutions having a pH of about 4. With this hydrogen ion concentration there was no apparent effect. Next a solution of pH 2 was tried. Contrary to what was expected this concentration caused a considerable rise in the first half-hour followed by a fall below normal during the second half-hour.

After the preliminary experiments had given this result more careful experiments were made. The first concentration to be used was pH 1.35. This gave a rise of 20 per cent during the first 20 minutes, which was followed by a fairly rapid fall below normal. The second concentration to be used was pH 1.95. The rise in this case was more gradual than in the preceeding one and the fall was also much more gradual and did not fall nearly so much. A few experiments were also made with pH 1.10. These were similar to those with pH 1.35 except that the decrease was sharper and greater. Solutions of pH 1.70 gave results very much like those with pH 1.95.

A large number of experiments were made with pH 2.65. These results were rather variable. With only a few exceptions the rate of respiration was always at or above normal during the entire experiment. The experiments that gave the most divergent results were rejected, and from the remainder an average curve was constructed, which for 80 minutes did not fall below normal (Figs. 1 and 2).

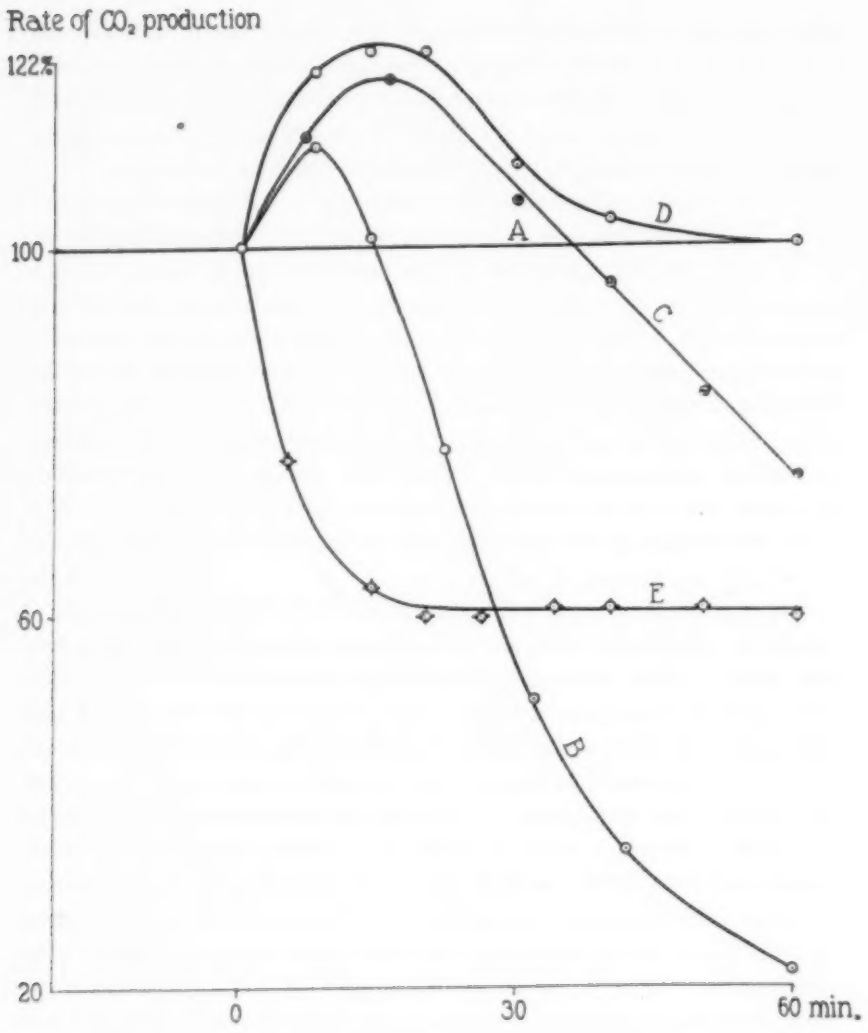

FIG. 1. Respiration of *Penicillium chrysogenum* expressed as per cent of the normal (at pH 7). The material was put in the test solution at the point marked 0 on the abscissa and the line at the left of this point represents the normal respiration. Curve A represents the respiration in a solution having a pH of 7 (normal); Curve B in pH 1.10; Curve C in pH 1.95; Curve D in pH 2.65; and Curve E in pH 8.80. The normal rate represents a change in the indicator tube from pH 7.94 to 7.52 in from 2 to 3 minutes depending upon the amount of material used. The probable error was less than 5 per cent of the mean, except at the last point with pH 1.95 where it was 6.5 per cent of the mean.

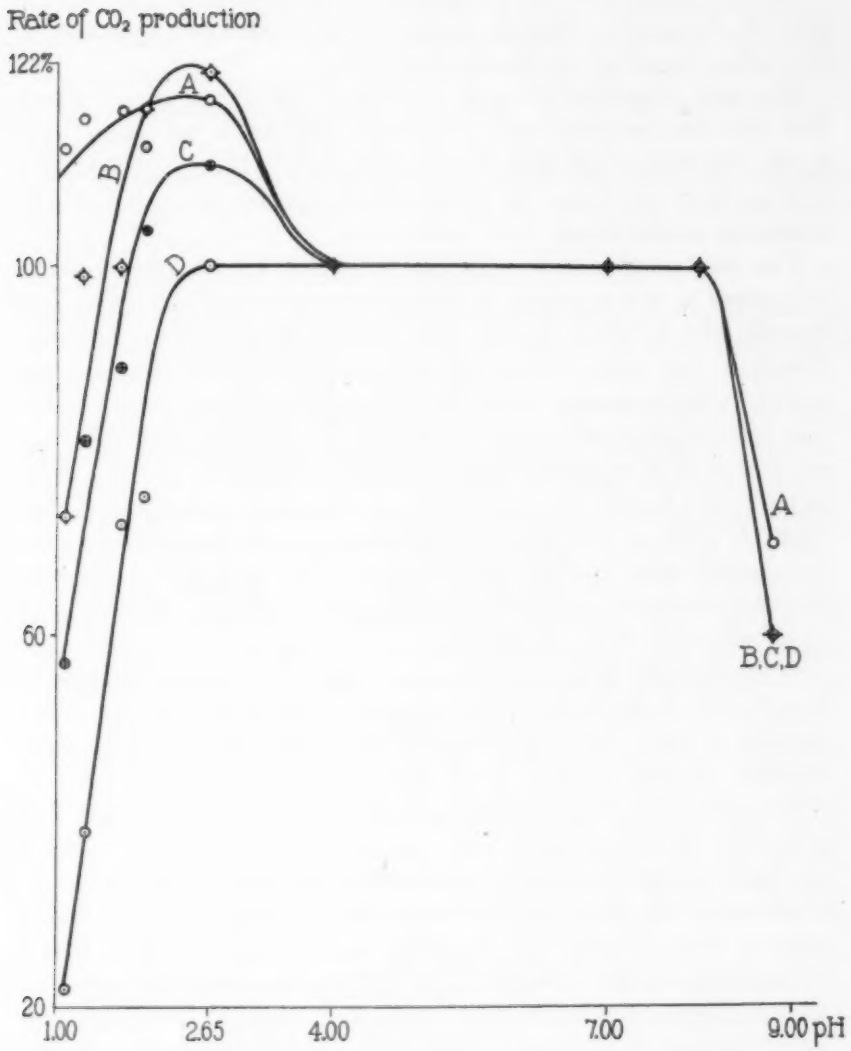

FIG. 2. Respiration of *Penicillium chrysogenum*. The curves show rate plotted against pH, and represent the same data as in Fig. 1. Curve A represents the respiration at the end of 8 minutes in the solution; Curve B at the end of 20 minutes; Curve C at the end of 30 minutes; and Curve D at the end of 60 minutes.

These experiments showed that in acid solutions the rate of respiration was increased at certain concentrations. The next step was to determine the effect of alkaline solutions.

The first concentration to be tried was pH 8. As it was found that this concentration had no effect on respiration the normal rate in the succeeding experiments was measured between pH 7.52 and 7.25 as the color change in phenolsulfonephthalein is much easier to determine at this range.

The next concentration chosen was pH 9. As phenolsulfonephthalein is not sensitive in this range phenolphthalein was used instead; this indicator is not toxic to the fungus. The concentrations were first standardized and it was found that the same amount of CO_2 caused a change from pH 7.52 to 7.25 and from 8.92 to 8.68. The time required for normal respiration to change the solution from pH 7.52 to 7.25 was determined; then the fungus was put in a solution slightly higher than pH 8.92 and the time required to change from pH 8.92 to 8.68 noted. It was found to be much longer than the normal, thus showing that respiration was decreased. Not only the first reading but all the succeeding ones showed a decrease, and at no time was there an increase.

When the pH value was less than 7 and there was a decrease to considerably below normal, no recovery back to normal (or nearly normal) followed, after the material had been replaced in ordinary nutrient solution at pH 4 or in sugar solution at pH 7. After the respiration had been depressed to 60 per cent of normal by pH 8.80 and kept there for an hour, there was nearly complete recovery when put back in sugar solution at pH 7. It is therefore evident that any considerable decrease produced by acid solutions is irreversible, while a similar decrease produced by alkaline solutions is reversible.

Some experiments have also been made by measuring the consumption of oxygen by the fungus. Winkler's method, as modified by Osterhout and Haas,[17] was employed.

It was found that in a solution of pH 9 the fungus uses less than one-half as much oxygen as in a neutral solution, while in a solution of pH 2 the consumption of oxygen is nearly four times as great as

[17] Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii, 141.

in a neutral solution. These experiments confirm the data obtained by measuring the production of CO_2, and show that in an acid medium the increase in oxygen consumption is greater than the increase in the production of CO_2, while in alkaline solution the two are about the same.

In all cases control experiments were made with solutions containing no fungus but having the same pH values as the solutions containing the fungus.

The results indicate that the increased production of CO_2 in acid solution is due to respiration and not to the action of the reagent in setting free CO_2 previously stored in the tissue (in the form of carbonates).

The work of certain investigators might lead to the expectation that, in general, moderate concentrations of acid would decrease respiration, while moderate concentrations of alkali would increase it. It is evident that the organism here studied behaves in the opposite manner. This raises some interesting questions regarding the reactions in the organism which result in the production of CO_2; further investigation will be necessary to clear up these questions. It is possible that the results obtained from the study of *Penicillium* are connected with the fact that it grows best on an acid medium. In the experiments of the writer spores grew best on media with a pH value of from 4 to 6.

SUMMARY.

1. Variations in pH value between 4 and 8 produce practically no effect on the normal rate of respiration (the rate at neutrality is called normal).

2. Increasing the pH value to 8.80 causes respiration to fall to 60 per cent of the normal, after which it remains stationary for the duration of the experiment.

3. Decreasing the pH value to 2.65 causes a gradual rise and a gradual return to normal; at pH 1.10 to 1.95 the preliminary rise amounts to 20 per cent and is followed by a fall to below the normal.

4. The decrease in respiration brought about by solutions of a pH value of 1.95 or less are irreversible, while a similar decrease

which occurs at pH 8.80 is reversible, the rate coming back to practically normal after the material is replaced in a neutral solution.

5. Determinations by means of Winkler's method showed an increase in the consumption of oxygen in acid solutions and a decrease in alkaline solutions.

THE ANALYSIS OF NEUROMUSCULAR MECHANISMS IN CHITON.

By W. J. CROZIER.

(From the Hull Zoological Laboratory, University of Chicago, Chicago.)

(Received for publication, May 3, 1920.)

From the standpoint of its grosser structure the nervous system of Placophorans is but poorly centralized, as evidenced by the absence of clearly defined ganglionic concentrations upon its principal strands. Histologically, the character of this nervous system is incompletely known. From the functional side, evidence has been secured pointing to the relatively diffuse type of nervous organization prevailing in Chiton as compared with some other mollusks (Arey and Crozier, 1919). It was desirable to make further inquiry as to the nature of the nervous organization of Chitons, especially as revealed through the actions of alkaloids. The species employed was *Chaetopleura apiculata* Carp, (found at Woods Hole, Massachusetts).

The neuromuscular responses of Chitons comprise movements of various parts of the body, resulting from localized stimulations, and movements of the animal as a whole. In the latter category are included locomotion, suctional attachment to a substratum, and curling up movements.

The locomotion of *Chaetopleura*, like that of other Chitons (*cf.* Crozier, 1918–19), is accomplished by neuromuscular waves which travel from anterior end to posterior end over the surface of the foot. In this species, as in other cases, one pedal wave at a time usually appears on the foot, although in the larger specimens (20 mm. in length and over), two or three successive waves may be seen on the foot simultaneously.

Chaetopleura is negatively phototropic when exposed to light of 150 or more meter candles intensity; to light of less than 150 meter candles the older individuals are indifferent. An animal creeping in bright light ceases movement when the light is removed, but the shell surface is not sensitive to a decrease of light intensity.

The strong suctional attachment of Chitons, following stimulation, is effected by the girdle; the flexible girdle is pressed into contact with the substratum, and a subsequent slight arching of the body serves to produce a true "sucker" action quite independent of the activity of the foot. The surface of the foot does, however, suck locally (Parker, 1914), an area several millimeters square being sufficient to afford a suctional field adequate for the support of the whole body. An undisturbed *Chaetopleura* is supported in this way, for the girdle is then lifted permitting the entrance of respiratory water currents.

A *Chaetopleura* detached from its substrate and placed on its back undergoes, in the light, a pill-bug-like curling up. In weak light or in darkness, the animal after a short time opens out, twists longitudinally, and may succeed in righting itself. The ventral surface is quite sensitive to light, and to shading. *Chaetopleura* attached to a glass slip may be caused to release the attachment of the foot by the illumination of the ventral surface. Either increase or decrease of light intensity causes a *Chaetopleura* lying on its back to curl up more completely. By means of such changes of light intensity, produced on the ventral surface through the animal's own movements while beginning to "open up" after being curled, a more or less rhythmic succession of curling contractions may be maintained. The posterior end of a curled up *Chaetopleura* is commonly more sharply bent than the anterior, which thus overlaps it (as noticed also by Sampson, 1895). The girdle musculature is not strongly contracted when the Chiton is curled up.

The degree of longitudinal curvature of the body of a Chiton, and the degree of elevation of the girdle are each controlled by the relative tensions of antagonistic muscle groups (for structure, *cf.* Sampson, 1895; Plate, 1901). The important muscles are: (1) a median dorsal group, composed mainly of fibers running from the anterior edge of each shell plate to the ventral surface of the anterior region of the next anterior plate; (2) longitudinal muscles at either side of the body (flexor antagonists of the extensor muscles (1)); (3) the dorsal (extensor) and ventral (flexor) muscles of the girdle, set perpendicular to its margin. The contact irritability of the foot and the activities of these muscle groups were studied especially in relation to the effects of strychnine, nicotine, and curare.

Strychnine.

Chitons attached to the bottom of a glass dish were covered with sea water, and later strychnine sulfate in sea water was added to the desired concentration; or else the animals were immediately covered with strychnine solution. The effects secured depended upon the strychnine concentration and upon the time of action.

In strychnine with a concentration of 1:10,000, after about 3 minutes (at 19°C.) the girdle of a Chiton begins to turn upward; the foot ceases to attach to the substratum, and the whole body curls upward at both ends; usually the animal falls over on one side, and finally comes to lie more or less on its back because of lack of stable support on the curved margin of the girdle; it later curls in the more usual fashion, with the dorsal surface convex. Typical waves, frequently several at a time, appear in rapid succession on the foot, while the body is bent; and the proboscis is greatly extended. At no time does the animal respond to a sudden shading of its dorsal surface, although for a brief interval reactivity to a shading of the ventral surface is greatly enhanced; shortly thereafter this kind of irritability is obliterated. From this moment, that is after about 5 minutes exposure to strychnine, the Chiton responds to a sudden *increase* of illumination upon its ventral surface by a very decided further uncurling—whereas, in the non-strychninized animal, such stimulation induces prompt curling. This reversal of the usual reaction to increased illumination persists for about 15 minutes if the Chiton is at this point returned to sea water; it is lost if the animal continues in the strychnine solution for 24 hours, but even in this case it reappears during subsequent destrychninization in sea water.

The initial phase of dorsalward (opisthotonic) curvature, due to the contraction of the median longitudinal and oblique dorsal muscle bands, is succeeded by the strong contraction of the ventral girdle muscles and of the lateral longitudinal muscles, so that the body becomes curled as in the usual curling up ma[illegible]ver. The dorsal shell plates are caused to separate slightly in [illegible] process, so that the intertegmental mantle between each two pl[illegible] is exposed and open to stimulation by touching it with a pointed instrument. In the unstrychninized Chiton local stimulation of the mantle between two

plates results in the approximation of these two plates, thus covering the spot irritated; this reaction is in nó way modified under strychnine (even at 1:4,000 concentration). The girdle, also, responds in an entirely normal fashion to local tactile activations, as do the ctenidia. Another form of response which remains unaltered under strychnine is the dorsalward contraction of the ctenidia against the roof of the ctenidial channel when a spot on the same side of the foot is touched. The surface of the foot itself exhibits an interesting modification of its responses; the foot ordinarily responds to the contact of a surface, sufficiently large with respect to the foot, by a positive suctional attachment (the adhesion of the foot being due to the activity of many minute suctional fields over its surface); but under strychnine, the foot puckers away from the contact of a surface of any size—its positive stereotropism suffers reversal. In the non-strychninized Chiton the touch of a fine-pointed rod causes merely a localized but rather deep puckering of the foot. Under strychnine, touching one small point leads to a widespread retraction.

With more concentrated solutions (1:4,000) these effects develop more promptly. The body becomes at first greatly arched, the back convex, with some convulsive trembling, but the girdle does not curl ventrally. If touched on the back, the animal usually ceases to adhere to the bottom and curls up, armadillo fashion, remaining so for some minutes. Ultimately, even in strychnine of low concentration (1:12,000), the Chitons pass into a paralyzed state, with all muscles contracted, the girdle curled ventralward, the two ends of the body more or less approximated ventrally.

The significant features of these results are: (1) The reversal by strychnine of the suctional attachment of the foot;[1] (2) the neuromuscular reversals exhibited in the dorsalward curvature of the girdle and of the body as a whole, probably due to the effects of impulses originating in those parts at the time in contact with the substratum; (3) the reversal of the response to increased illumination of the ventral surface; (4) the fact that certain responses seem to remain

[1] This is paralleled by Moore's (1916–17) finding with the tube feet of strychninized starfish.

relatively uninfluenced by strychnine (movements of the ctenidia, and of the dorsal plates when the intertegmental mantle is touched).[2]

In the strychninized Chiton sensory impulses originating on the ventral aspect of proboscis, foot, and girdle may be regarded as causing contraction of *extensor* (dorsal) muscles of the girdle and of the body (*median dorsal longitudinal* and *oblique dorsal muscles*). This effect may be accentuated by touching the back of the Chiton. Such impulses normally lead to the contraction of *flexor* muscles (*ventral* muscles of the girdle; *lateral longitudinal muscles* connecting the shell plates) which results in a closer contact between the Chiton and its substratum.

Reversal of the response to increased illumination of the ventral surface, involving the muscle groups mentioned, operates in the same manner. This seems to be the first recorded instance of experimental reversal of the sense of a response dependent upon "differential sensitivity."

There is a detectable degree of independence in the movements of strychninized girdle and body. Thus at certain stages a touch on the back may cause the girdle to bend ventrally, while the extensor muscles of the back contract; in other instances the back may be arched, while the girdle bends dorsally. The type of local action here indicated is further illustrated by the behavior of Chitons with the pallial nerve strands cut and with the proboscis region isolated from the foot by a transverse cut. Such preparations do not differ noticeably from intact animals in their responses to strychninization.

[2] In a number of instances Chitons returned to sea water after having been in strychnine solution for a number of hours were found to pour out great volumes of sperm, at a certain stage in the progress of recovery. The observation is of interest in connection with the view (Crozier, 1920) that the shedding of sperm by male Chitons, although in normal fertilization preceding the liberation of eggs, is in reality induced as a sensory response to substances emanating from females; in these experiments the proper internal concentration of strychnine for the release of the nervous machinery controling sperm discharge seems to have been established during the outward diffusion of the drug from the Chiton into sea water.

Nicotine.

Attached *Chaetopleura* immersed in 1:25,000 solutions of nicotine (Kahlbaum) within 3 minutes release their suctional attachment to a surface, and curl ventralward rather sharply at both extremities. The proboscis fold is commonly much protruded, covering the anterior end of the foot. The girdle is curled inward. In this tightly curled condition the Chiton quickly passes into paralysis. After 15 minutes in nicotine solution, recovery of normal movements is effected within an hour following return to sea water. At 1:1,000 concentration the nicotine effect is almost instantaneous. After the first few seconds in nicotine solution the ordinary responses to changes in illumination are obliterated.

Nicotine primarily induces the contraction of *flexor* muscle groups (ventral girdle muscles, lateral shell muscles), whereas in the early stages of strychninization the extensor antagonists of these muscles are the most readily contracted.

A further peculiarity of the nicotine action consists in its selective nature. The anterior end of the Chiton is almost invariably more strongly flexed than the posterior. When curled up in the absence of nicotine, the posterior end is the more flexed, the anterior end folding over it. When the body has been partially transected in such fashion that the soft tissue of the foot is separated from the proboscis fold, while the dorsal muscle bands are left in continuity, it is found that nicotine induces as promptly as before the general flexion of the girdle, while the foot and shell muscles remain for a long time unaffected. The action of strychnine on such a preparation, already noted, is quite different. Thus the "cerebral" region of the nervous system of Chiton, despite the absence of pronounced cephalization, exhibits nevertheless a certain degree of selective reactivity with nicotine, homologous with that demonstrated by Moore (1918-19) for the cerebral ganglia of the squid.

Curare.

Immersion of a Chiton for 3 hours in a saturated emulsion of active curare has practically no detectable results on the animal's movements. Detached and partially curled up *Chaetopleura* dropped

into curare solution tend on the whole to remain curled up for a longer period (even in the dark) than is the rule in sea water, but, even after 5 hours in curare, recovery in sea water is quite prompt. The isolated foot of a Chiton, if exposed to curare, does not go into spasms of contraction. Curare has a quite different effect on the foot and on the whole animal in the case of gastropods, usually inducing convulsive contractions. Even after 7 hours in curare the tendency to curl up is not materially enhanced; the foot is usually crinkled somewhat, but not decidedly, and the girdle is not strongly contracted. It is of interest to note that curare has practically no action on neuromuscular coördination in flatworms.[3]

In comparison with the definite curvatures of body and girdle characteristically induced by nicotine and in early stages of strychninization, it is found that in quinine solutions the curvature of the mantle is quite irregular, while the body may be curled to varying degrees.

SUMMARY.

1. The degree of curvature of the body and of the girdle of a Chiton is determined by the activity of antagonistic muscle groups. At a certain early stage in the strychninization of a Chiton the reciprocal inhibition involved in the natural use of these muscle groups is reversed, such that extensor muscles, rather than, as normally, flexor muscles, contract as the result of stimulation. This condition involves a reversal, under strychnine, of the normally positive stereotropism of the foot, and of the usual response of the mollusk to an increased illumination of its ventral surface. Strychnine reversal of this character is not a matter of the relative strength of the opposed muscle groups, for the flexor muscles are the more powerful and are the ones always shortened in tetanic contraction.

2. Nicotine, in contrast to strychnine, primarily induces contraction of flexor muscles. Its effects, moreover, are in a degree selec-

[3] Vles (1907) thought the antero-posterior direction of the pedal waves in *Acanthochites* suggestive of a worm-like neuromuscular organization, more primitive than that in the foot of gastropods, where the waves are often *direct*; but the retrograde waves producing locomotion in polyclads (Crozier, 1918) seem to be rather more complex than in Chitons (Crozier, 1918–19).

tive, being notably exerted on "cerebral" nervous structures. Curare is devoid of characteristic action on the neuromuscular responses of Chiton.

3. The chemical organization of the neuromuscular organs of Chiton, as far as revealed by these tests, corresponds to a more simple condition than is inferred for gastropods. In particular, the behavior with respect to curare resembles more that of the neuromuscular apparatus of flatworms.

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THE KINETICS OF THE ACTION OF CATALASE EXTRACT FROM MARINE ALGÆ, WITH A NOTE ON OXIDASE.

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There exists, as far as we know, no literature on the catalase of marine algæ. The work done on oxidase and peroxidase is scanty and incomplete (Reed, Atkins, and Duggar and Davis). We have started a preliminary investigation of the catalase, peroxidase, and oxidase of marine algæ. The present paper deals only with the enzymes of *Ulva tæniata* Setchell and Gardner, although we worked with different forms. *Ulva* contains the strongest oxidase, according to Reed. This fact, and the abundant occurrence of *Ulva* at Pacific Grove, led us to study this form more particularly.

Our work is only preliminary, for as yet we have not considered the daily variations and other features of the behavior of the enzyme. We hope to publish soon a more physiological paper on the same question. We worked with one "clan" of *Ulva*, placed in an aquarium with running sea water, the temperature of which was practically constant throughout our experiments. We always worked upon the youngest plants, but the "physiological error" will remain in our work a source of inevitable inaccuracies.

Catalase.

General Remarks.—The alga contained a rather active catalase, as qualitative experiments have shown. To determine its strength we used a manometrical method, elsewhere described by us. A suction flask was connected with a self-recording manometer by a ground joint. This joint permitted thorough shaking of the flask. The suction flask was closed by a ground stopper to which the peroxide container was sealed on the inside. On inverting the flask, the peroxide would pour into the fluid in the flask and the reaction would

begin. The curves made on millimeter paper by the automatic writer could be calculated immediately. The advantages of this method are: (1) accurate determination of the beginning and the end of the reaction and of the reaction *time*; (2) accurate determination of the strength of the peroxide; (3) accurate and quick measurement of reaction velocities in very short intervals; *e.g.*, every 5 seconds.

Fig. 1 is an autogram showing the effect of the action of 3 cc. of 3 per cent peroxide on 3 gm. of ground *Ulva* thallus in a slightly alkaline medium. The ordinates are pressures in millimeters. The abscissæ are 14 seconds per millimeter. We used Braun-Knecht-Heimann's C. P. 3 per cent peroxide which contains still acetanilide and acid. We neutralized the peroxide with 0.1 N sodium carbonate solution.

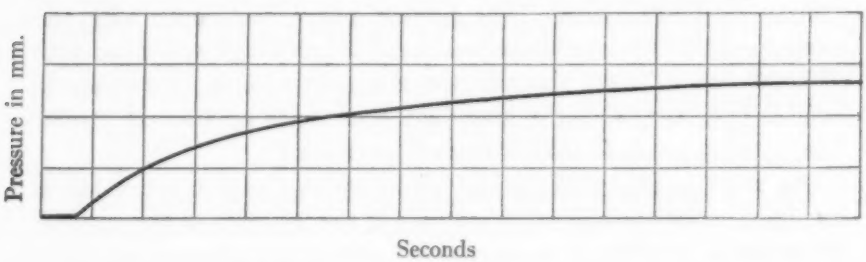

FIG. 1. Autographic record of the effect of 3 cc. of 3 per cent peroxide on 3 gm. of fresh, ground thallus of *Ulva*.

The activity of the catalase in *Ulva* is not very great. If we compare it with the activity of hemp leaves, we get

3 gm. of ground *Ulva*.....4 cc. of H_2O_2 + 0.4 cc. of 0.1 N Na_2CO_3
Average.....$K = 521 \times 10^{-5}$
3 gm. of ground hemp leaves...4 cc. of H_2O_2 + 0.4 cc. of 0.1 N Na_2CO_3
Average.....$K = 7{,}020 \times 10^{-5}$

The *hemp* is about fourteen times stronger than the *alga* in its catalytic activity.

Before giving a detailed description of the experiments, we wish to emphasize the fact that, however simple the method described may seem, we met with great difficulty in carrying it out. We discarded most of our experiments because we were not sure of their

reliability. Only smooth, finished curves were used for calculation. These were produced in experiments in which the ground plant was mixed thoroughly with the peroxide. In connection with these remarks a quotation of Pickering is given:

"The application of the graphic method requires a great amount of care and close attention to experimental and other conditions, and it is to be feared that hurried use of it by those who have not taken the trouble to master the necessary details, or to acquire the requisite amount of skill, may bring it into undeserved disrepute."

It is almost unnecessary to say that we used for our calculation the formula of van't Hoff

$$K = \frac{1}{0.434t} \log \frac{a}{a - x}$$

In the few cases to which this formula did not apply we used others. All the determinations were made at ± 17°C.(16–18°C.). The temperature of the sea water was invariably 15°C.

Catalase of the Undamaged Plant.—Catalase is an intracellular enzyme and is supposed to adhere to the cells with great tenacity. The extent to which the material is ground has a marked effect upon the result. Long and thorough grinding in a mortar reduces the alga to the consistency of an unguent. With the material in this condition we obtained our best results. At first we washed off the adhering sea water with distilled water before the grinding, thinking that the salt water might injure the enzyme. We were very much surprised, however, to find that these preliminary washings reduced the enzyme strength one-half, whereas a treatment with sea water had no effect at all.

3 gm. of *Ulva* + 3 cc. of unneutralized peroxide, ground without washing, gave the following.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
275	270	256	254	264

K decreases because of the acidity of the peroxide.

3 gm. of *Ulva* + 3 cc. of unneutralized peroxide, ground after washing twice in distilled water, gave the following.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
128	110	86	81	101

K decreases because of the acidity of the peroxide.

We could offer two explanations of this fact: (1) The distilled water destroys the enzyme. (2) The enzyme migrates into the wash water. To decide which hypothesis was true, we made a catalase determination with the wash water. We soaked 20 gm. of *Ulva* in 200 cc. of distilled water for 40 minutes. To 4 cc. of this wash water we added 3 cc. of peroxide solution. The wash water contained catalase.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
40	33	31	26	33

On the assumption that there is proportionality between K and enzyme quantity for 3 gm. of plant, calculation gave a yield of catalase of 130×10^{-5} as average K to the distilled water. We can say, therefore, that the plant lost one-half its catalase to the surrounding water. We obtain

Washed plants: $K \times 10^5$	= 100
Wash water	= 130
	230

Unwashed plants had a $K \times 10^5$ of 264 which is a fairly close approximation. After 1 hour and 40 minutes washing, the wash water contained no more catalase than after 40 minutes. The curve obtained was identical with the curve analyzed above. Wash water, in which the plant remained for only 3 minutes, showed no catalytic activity.

We then put 20 gm. of plants from the aquarium in 25 cc. of distilled water and let it stand over night. The next morning 4 cc. of the fluid gave

$K \times 10^5$ after.				Average K.
138 sec.	276 sec.	414 sec.	552 sec.	
139	109	96	85	107

which gives a yield of catalase of $K \times 10^5 = 85$ from 3 gm. of plants. The source of the above tables is the autographic record shown in Fig. 2.

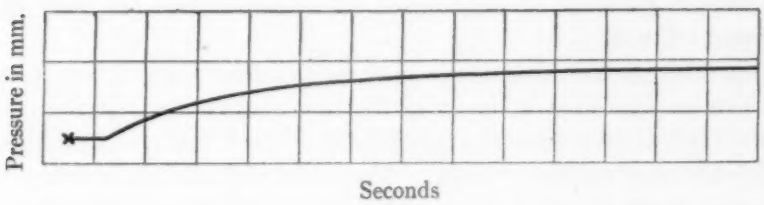

FIG. 2. Autographic record of presence of catalase in distilled water used to wash *Ulva*.

The enzyme is more or less destroyed by long standing, and we can conclude with certainty that approximately *one-half the total amount of catalase passes out into the wash water within the first 40 minutes of washing*.

There is, as far as we know, only one paper dealing with a similar question. Lyon has noted similar behavior with echinoderm eggs. He found that in these eggs the catalase activity was doubled after fertilization. The increase in activity started about 3 minutes after fertilization. Lyon discusses several possible causes of this fact and finds that neither the sperm nor the "increase in activity" is responsible for the change but on the other hand a difference in the permeability of the cell membrane. This is consistent with the theory of fertilization set forth by Loeb, Warburg, and others. Lyon was able, by placing the echinoderm eggs in distilled water, to *double the catalase activity in unfertilized eggs*; in other words, they gave the same reaction as the fertilized eggs. Lyon seems not to be aware of

the fact that the catalase was suspended in the water. In connection with our own experiments we tried to determine the facts microscopically.

Experiment 1.—The damaged piece of thallus was placed in sea water + a trace of H_2O_2. Bubbles of oxygen originated only at the place where the piece was cut. After a long time there was a very feeble activity on the surface ceasing after about 2 hours.

Experiment 2.—A similar piece of thallus was placed in distilled water + a trace of H_2O_2. Oxygen originated immediately at the place where the piece was cut. After about 3 minutes there was a strong reaction on the whole surface, which lasted for about 50 minutes.

There are two conclusions possible, which might explain these facts: (1) catalase goes out by exosmosis; (2) catalase goes out through the burst cell wall.

While the second assumption may be possible, there is one fact that can be explained in favor of the first assumption. If the cell bursts, there is no reason why any catalase should remain in the cell, as the enzyme is soluble in water. A fact that reminds one of exosmosis is the short space of time within which the emigration of the catalase is completed. It is improbable, on the other hand, that large molecules diffuse out of the cells in which they occur. We may suspect, therefore, that this enzyme at least is a simpler compound than most of us have thought hitherto.

Influence of Substrate Concentrations.—We considered one series of experiments done with homogeneous material under the same conditions. 3 gm. of *Ulva* were ground in a mortar to a very fine consistency with 15 cc. of distilled water. The peroxide was previously neutralized by 0.1 cc. of 0.1 N sodium carbonate solution per cc. We found

Peroxide.	End-pressure.	$K \times 10^5$ after.					Average. $K \times 10^5$	Reaction time.	
		138 sec.	276 sec.	414 sec.	552 sec.	690 sec.		Found.	Calculated.
cc.	*mm.*							*sec.*	*sec.*
4	44	306	298	295	299	306	299	745	744
3	32	342	333	353	366	361	337	573	558
2	21	438	408	461	514 (?)	437	423	372	372
1	10	667	689	—	—	—	678	207	186

Peroxide quantities are proportional to reaction time, as the last two columns show. No linear relation exists, however, between the former and the reaction velocity. Fig. 3 shows that this relation can be expressed by a continuous curve which is not exponential, as the following calculation indicates.

Peroxide.		K^1	$K^{1.5}$	$K^{1.8}$	K^2
cc.					
4	$\frac{K}{4}$	100	100	100	100
3	$\frac{K}{3}$	56.5	57.4	62.9	64
2	$\frac{K}{2}$	47.1	57.4	62.2	65
1	$\frac{K}{1}$	53.1	83.1	107	123

There seems to be no simple connection between peroxide quantity and reaction velocity. The laws derived by Schutz-Borissow, Issajew, and Herzog do not hold here.

This is otherwise with enzyme quantity. Three curves, run with 3 cc. of neutralized peroxide and different amounts of enzyme gave

Enzyme.	$K \times 10^5$	$K \times 10^5$ Calculated.*
2 gm. of plant.	256	254
3 " " "	380	380
4 " " "	506	508

* K calculated after 414 sec.

According to the low strength of the enzyme, the peroxide is very much in excess, and therefore the reaction velocity is proportional to the enzyme concentrations, not to the peroxide concentrations. Senter found exactly the same to be the case with blood catalase (see also the more recent paper by Yamasaki). The values obtained with hemp gave a proportionality to both peroxide and enzyme quantity. This is probably due to the great strength of the hemp catalase. However, it is remarkable that the reaction velocity still remains in a linear relation to the peroxide quantities. We have no

doubt that this relation will exist also with different enzyme quantities. Reaction time is a very valuable index for enzyme determination, and the fact that Duclaux and Went are the only authors who used this index in physiology shows us that it is very little known. The catalase strength is often determined by measuring a pressure after a certain time, but this method has no value for quantitative determinations.

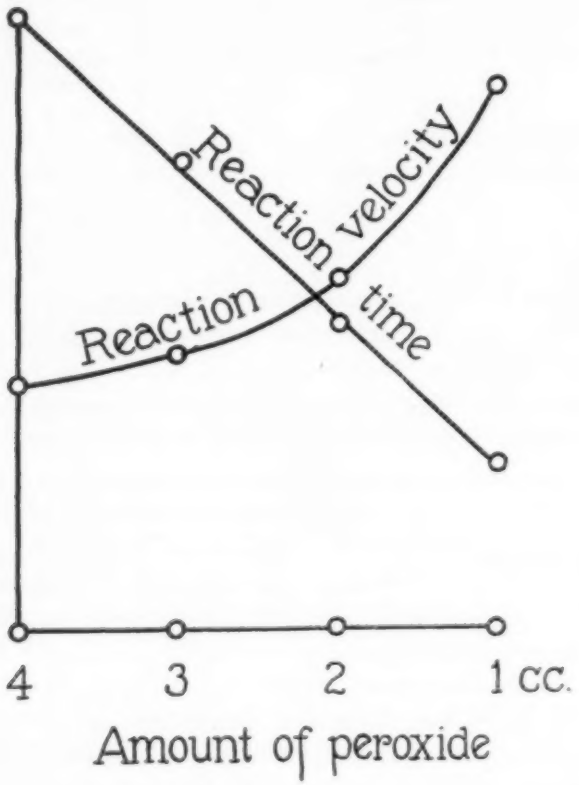

FIG. 3. Relations of peroxide quantity, reaction time, and reaction velocity.

Influence of External Conditions.—Under certain precautions the reaction follows the monomolecular line. For example, 3 gm. of *Ulva* were ground as fine as possible in a mortar with 15 cc. of distilled water. After reaction with 4 cc. of peroxide and 0.4 cc. of 0.1 N Na_2CO_3 solution we calculated the resulting curve.

Time.	$a-x$	$\log \frac{a}{a-x}$	$\frac{2.31}{t} \log \frac{a}{a-x}$
sec.			
138	29.0	1.183	306×10^{-5}
276	19.5	0.353	298×10^{-5}
414	13.0	0.527	295×10^{-5}
552	8.5	0.715	299×10^{-5}
690	5.5	0.904	306×10^{-5}
828	3.8	1.063	296×10^{-5}
966	2.4	1.272	304×10^{-5}
Average.....			$(300 \pm 9) \times 10^{-5}$

$a = 44$ mm.

The reading error, 0.2 mm., gave the following result in the first K:

138 29.2 0.178 297×10^{-5}

The deviations in K are therefore due to reading errors, and the reaction follows the monomolecular line.

We studied the influence of different amounts of sodium carbonate with different 3 gm. lots of *Ulva*. We found

	$K \times 10^5$ after.				Average $K \times 10^5$
	138 sec.	276 sec.	414 sec.	552 sec.	
3 cc. of H_2O_2 + 1.6 cc. of Na_2CO_3 (neutral to phenolphthalein).....	338	356	360	349	351
3 cc. of H_2O_2 + 100 cc. of Na_2CO_3 (alkaline)..	251	219	215	176	215
3 cc. of H_2O_2 + 1,000 cc. of Na_2CO_3 (alkaline)..	165	155	151	149	155

Excess of alkali causes a marked decrease in reaction velocity in subsequent repetitions of the reaction. Perhaps the enzyme is destroyed by the carbonate. Therefore we tried to apply the formula of Schmidt-Nielsen (shaking in activation of rennet) $\frac{dx}{dt} = k(a-x)^{\frac{3}{2}}$ in the modification given by Herzog

$$K = \frac{\sqrt{a} - \sqrt{a-x}}{t\sqrt{a\,(a-x)}}$$

	$\sqrt{a}$	t	$\sqrt{a-x}$	$\sqrt{a(a-x)}$	$K \times 10^5$
3 cc. of H_2O_2 + 100 mg. of Na_2CO_3.	5.25	276	3.87	20.4	245
		414	3.39	17.9	251
		552	3.00	15.8	254
		690	2.74	14.4	256
		828	2.55	13.4	244
		966	2.34	12.4	243
Average.....					$(249 \pm 7) \times 10^{-5}$
3 cc. of H_2O_2 + 1,000 mg. of Na_2CO_3.	5.25	276	4.18	21.9	177
		414	3.8	19.9	176
		552	3.47	18.3	174
		690	3.16	16.6	182
		828	2.95	15.7	177
		966	2.72	14.3	184
Average.....					$(178 \pm 7) \times 10^{-5}$

So we see that the formula of Schmidt-Nielsen is valid for the case of excess carbonate.

Unneutralized peroxide was harmful, as the following experiment shows.

3 gm. of *Ulva* + 3 cc. of unneutralized H_2O_2 and 15 cc. of distilled water.

$K \times 10^5$ after.				Average. $K \times 10^5$
138 sec.	276 sec.	414 sec.	552 sec.	
275	270	257	256	265

The summary of this experiment is given in Fig. 4. The point 0, in the figure, "neutral to phenolphthalein," seems to be the optimum point for catalase. (For similar lines see Bredig and von Berneck and Jacobson's work.)

As described above, the water in which undamaged plants remained for a certain time contained catalase. This catalase did not follow the logarithmic curve.

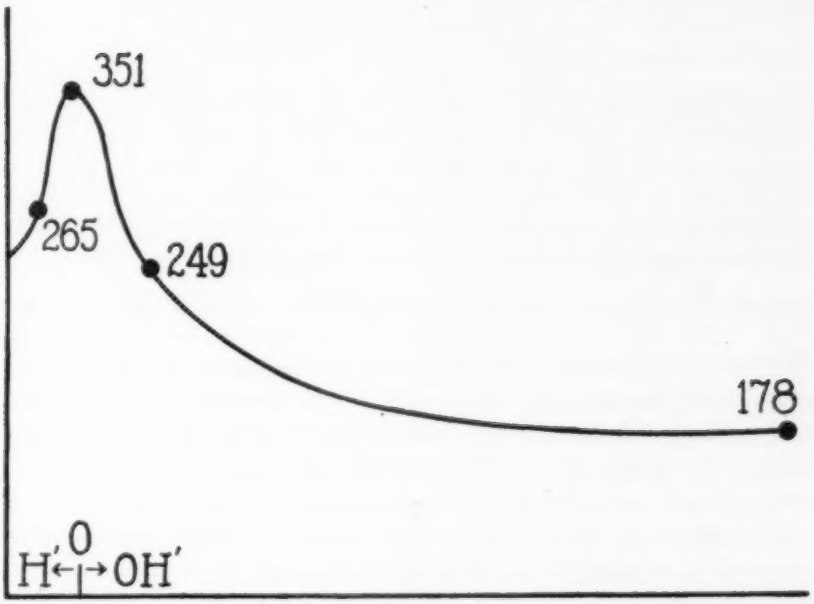

FIG. 4. Graphic indication of the reaction velocities with varying amounts of Na_2CO_3.

	t	$K \times 10^5$	$K^2 \times 10^5$	$K^2 \times 10^5$	$K^3 \times 10^5$
20 gm. of plant in 25 cc. of water.	138	139	134	130	129
	276	109	109	117	123
	414	96	99	101	120
	552	85	91	95	126
Average.....					$(124 \pm 5) \times 10^{-5}$

Calculation following Schmidt-Nielsen's formula (K as in Column 4) gave no better result. Next we tried $\frac{dx}{dt} = K(a - x)^2$ (as in Column 5) and $\frac{dx}{dt} = K(a - x)^3$ (as in Column 6). The latter gave a constant series. The destruction of the enzyme is probably due to other causes (substances washed out by exosmosis (?)).

	t	$K^1 \times 10^5$	$K^2 \times 10^5$	$K^3 + 10^5$
20 gm. of plant in 200 cc. of water.	138	40	38	43
	276	33	35	38
	414	31	33	35
	552	26	29	31

However, this assumption does not hold, as the above table shows. Here the dilution is eight times greater than in the former case. The formula $\frac{dx}{dt} = K(a - x)^3$ does not apply here. It is obvious that the *dilution* is the factor which combines its injurious effects with the chemical influences. Herzog remarks that inactivation of enzymes is the quickest in dilute solutions according to the principle of Willard Gibbs. It is known in breweries that the diastase is resistant in concentrated malts, but is quickly destroyed in dilute malt.

Conclusions as to Catalase.—Ulva tæniata Setchell and Gardner contains a catalase which adheres to the cells to a certain extent, but may, mechanically or by exosmosis, pass into the surrounding medium. This catalase is able to act in a neutral medium on peroxide, the reaction being monomolecular. In an alkaline medium the catalase decomposes peroxide following the formula of Schmidt-Nielsen

$$K = \frac{\sqrt{a} - \sqrt{a - x}}{t\sqrt{a\,(a - x)}}$$

The reaction time varies in a linear relation with the concentration of the peroxide. Reaction velocity and enzyme concentration vary in a linear relation. Reaction velocity and peroxide concentration vary in a continuous non-linear and non-exponential relation. Since the peroxide is in excess of the feeble catalase, these facts are in agreement with the data of Senter on blood catalase. On dilution the enzyme is destroyed, following highly exponential lines

$$\frac{dx}{dt} = K\,(a - x)^3$$

or even

$$\frac{dx}{dt} = K\,(a - x)^4$$

Oxidase and Peroxidase.

Oxidase.—Pyrocatechine is quickly oxidized in the presence of ground *Ulva*. 10 gm. of grindings, 200 mg. of pyrocatechine, and 25 cc. of water gave in 20 hours in the manometer a negative pressure of 2 mm. 5 gm. of grindings, 300 mg. of pyrocatechine, and 25 cc. of water gave in 20 hours in the manometer a negative pressure of 3 mm. These are indications that *Ulva* contains an oxidase. Reed found in *Ulva* an oxidase which acted on guaiacol, pyrogallol, hydrochinon, paraphenylendiamine, and α-naphtol.

To determine whether the reaction of the pyrocatechine was due to the action of the plant we prepared two test-tubes; A, 100 mg. of pyrocatechine and 10 cc. of distilled water, and, B, 100 mg. of pyrocatechine, 5 cc. of distilled water, and 5 cc. of *Ulva* extract. B gave a strong red color after 1 hour; A remained light yellow.

We were not able to get an appreciable reaction with guaiacol, paraphenylendiamine, or α-naphtol, or a combination of the last two substances.

100 mg. of pyrocatechine were oxidized in 17 hours by 1 cc. of extract to the same color as 0.2 cc. of 0.1 N potassium permanganate would give it.

100 mg. of pyrogallol were oxidized in 17 hours by 1 cc. of extract to the same color as 1.75 cc. of potassium permanganate would give it (dark brown). A control experiment without enzyme gave a light color (0.25 cc. of potassium permanganate).

100 mg. of guaiacol or 100 mg. of phenol were not oxidized in 17 hours.

In conclusion we may say that *Ulva* contains a rather strong oxidase, active on pyrocatechine and pyrogallol, inactive on guaiacol, phenol, paraphenylendiamine, and + α-naphtol.

Peroxidase.—We tried two peroxidase tests. One, the luminescence of pyrogallol + H_2O_2 (Harvey) failed under the different circumstances. The other, the action of plant juice on ring compounds + H_2O_2 succeeded only in a faintly alkaline medium. *We were able to obtain the same reactions without enzyme in alkaline solution.* Therefore, we do not feel justified in concluding that a peroxidase is present. For example

200 mg. of hydrochinon + 3 cc. of H_2O_2 give a feeble red color.
200 " " " + 0.4 cc. of 0.1 N Na_2CO_3 give a yellow color.
200 " " " + 3 cc. of H_2O_2 + 0.4 cc. of Na_2CO_3 give a strong orange color.

Addition of plant juice has no apparent effect. Similar results were obtained with paraphenylendiamine, pyrocatechine, and resin of guaiacol.

In connection with this fact we may mention the work of Welter on the peroxidase of tea. Welter found that sand (sea sand of Merck, purified) + H_2O_2 gave a peroxidase reaction with ring compounds.

SUMMARY.

Ulva tæniata contains oxidase. The existence of peroxidase is not proved.

We wish to express our gratitude to Dr. Walter Fisher, Director of Hopkins Marine Station of Stanford University, for his hospitality and valuable help, and to Dr. N. Gardner, of the University of California, for determination of the material with which we worked.

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QUANTITATIVE LAWS IN REGENERATION. II.

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I.

In a preceding paper[1] it has been shown that, when a piece of stem inhibits the production of roots and shoots in a leaf of *Bryophyllum calycinum* connected with it, the stem gains in mass and this mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. On the basis of this fact it was suggested that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available for this process naturally flows into the stem.

In these experiments the quantity of roots formed had not been measured directly but had been calculated on the assumption that the dry weight of the roots formed is on the average 42 per cent of the dry weight of the shoots formed in the same leaves. Since this experiment seems to be crucial for the answer to the question why the leaf does not form shoots or roots as long as it forms a part of a normal plant it seemed advisable to make a direct determination of the mass of roots formed by an isolated leaf. Five new sets of experiments, as a rule with eight pairs of sister leaves, were made. The method was the same as that described in the first paper.[1] Table I gives the dry weight of the organs. The experiments lasted about 1 month; if they last too short a time the error in measuring vitiates the result and if we wait too long another complication arises inasmuch as the leaves of the shoots formed become too large and contribute too much material for further growth and regeneration.

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297.

In Table I, a is the dry weight of roots and shoots formed by the isolated leaves without stems; b is the dry weight of the half stems cut off from these leaves at the beginning of the experiment; a_1 is the dry weight of roots and of shoots formed by the sister leaves left in connection with a piece of half stem. b_1 is the dry weight of the latter half stems at the end of the experiment. According to our theory the inhibitory effect of this piece of stem left in connection with a leaf on the production of roots and shoots by the leaf should be due to the fact that naturally the material (or the greater part of

TABLE I.

Dry Weight of Roots, Shoots, and Half Stems.

Experiment No.	Duration.	a		b	a_1		b_1	$\frac{a}{a_1 + b_1 - b}$
		Roots.	Shoots.		Roots.	Shoots.		
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	30	0.169	0.412	0.474	0.039	0.067	1.054	$\frac{581}{686} = 0.85$
2	33	0.133	0.408	0.426	0.062	0.158	0.753	$\frac{541}{547} = 0.99$
3	33	0.127	0.509	0.415	0.045	0.114	1.034	$\frac{636}{778} = 0.82$
4	30	0.127	0.438	0.563	0.053	0.143	1.029	$\frac{565}{662} = 0.85$
5	30	0.101	0.416	0.422	0.043	0.162	0.823	$\frac{517}{606} = 0.85$

the material) used for root and shoot production in the leaf flows into the stem and is utilized here for growth. We should therefore expect to find that the dry weight of the half stem (b_1) left in connection with the leaf should increase as much as or even more than the difference in the dry weight of the roots and shoots produced in the two sets of sister leaves amounts to. If a is the mass of shoots and roots produced by the leaf isolated completely from the stem, a_1 the mass of roots and shoots produced by the sister leaves left in connection with the half stem, b the mass of the half stems at the beginning of the

experiment, and b_1 the mass at the end, we should expect to find that $b_1 - b \lesseqgtr a - a_1$ or that $\frac{a}{a_1 + b_1 - b} \lesseqgtr 1$. Table I shows that this is correct. The average ratio of $\frac{a}{a_1 + b_1 - b}$ is 0.87.

When a leaf is connected with a stem which prevents regeneration in a leaf the inhibitory effect is therefore due to the fact that the material available for the regenerative growth in the notches of the leaf flows into the stem. In fact 14 per cent more dry weight goes from the leaf into the stem than would go into the growth of the regenerating leaf. For some reason the flow of sap into the regenerating parts in the notches of a leaf seems to be less complete than the flow of available material into the stem.

II.

The material which goes into the stem is used for different purposes; it may cause the growth of an axillary shoot in the stem as in Fig. 1. Part of it causes an increase in the mass of the stem. The stippled lines in Fig. 1 indicate part of the increase in the mass of the stem. This increase is greatest at the basal end where it constitutes the callus.

In a paper previously published[2] the writer has called attention to the fact that when a piece of stem is left in connection with a leaf the inhibitory effect on the formation of roots in the leaf increases with the size of the piece of the stem but less rapidly than this. This is due to the fact that the increase in mass of the stem is not the same throughout the whole length of the stem but that it is greater at the base and possibly also (though to a lesser degree) in the region of a node.

Hence the increase in mass of a stem consists of the sum of two quantities, $c + l\,c_1$, where c expresses the mass of the callus which does not necessarily increase with the mass of the stem, and $l\,c_1$, where l is the length of the stem and c_1 the increase in the mass per cm. length of a stem with unit periphery. It is obvious why the inhibitory power of a stem increases generally with its length but less rapidly.

[2] Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

Figs. 2 and 3 show the distribution of the reddish pigment formed in the leaves suspended in air and drying out. The reddish pigment is indicated by the stippled area in the leaf. In the two leaves connected with a piece of stem where the regeneration of shoots in the leaf is inhibited the pigment flows into the stem and the newly forming shoots in the stem. The old leaf contains pigment only in that part which is close to the petiole and this is obviously pigment in

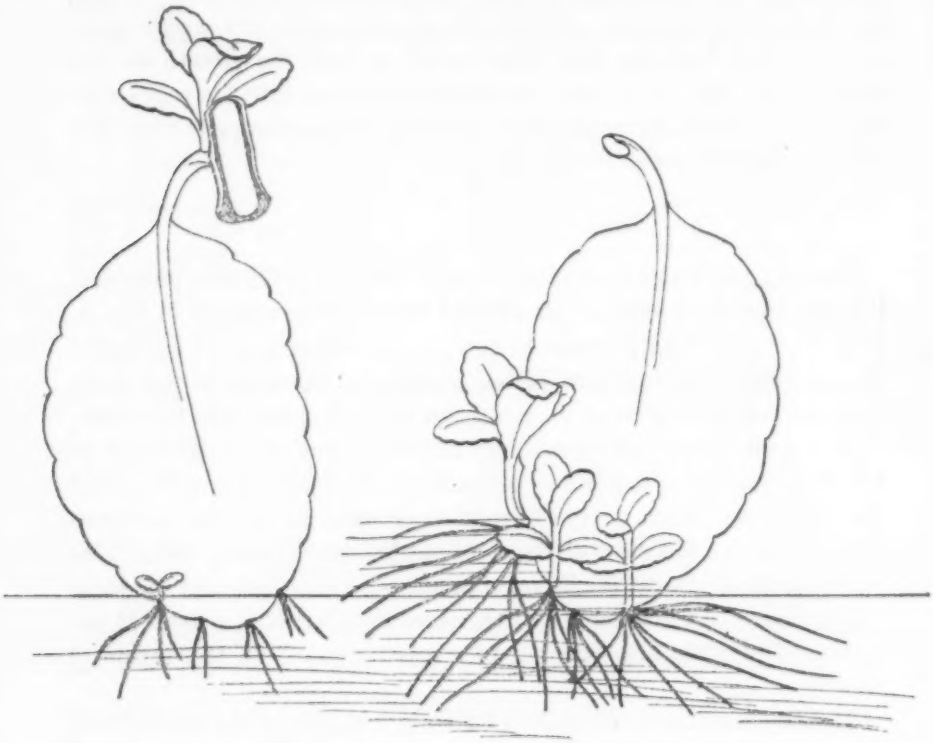

FIG. 1. Leaves suspended with apex in water. Inhibitory influence of a piece of half stem on shoot and root formation in leaf connected with it. The leaf to the right (without stem) has formed three vigorous shoots and numerous roots. The sister leaf with a small piece of split stem attached has formed in the same time a tiny shoot and a few roots. The material required for root and shoot formation in the leaf has migrated into the stem and gives rise there to an axillary shoot and to the growth in the stem indicated by stippling. Duration of experiment, Mar. 30 to Apr. 27.

the process of flowing into the stem. The two sister leaves without stems have formed shoots and roots in their notches, one at the base and the other on one side. The reddish pigment is collected near the

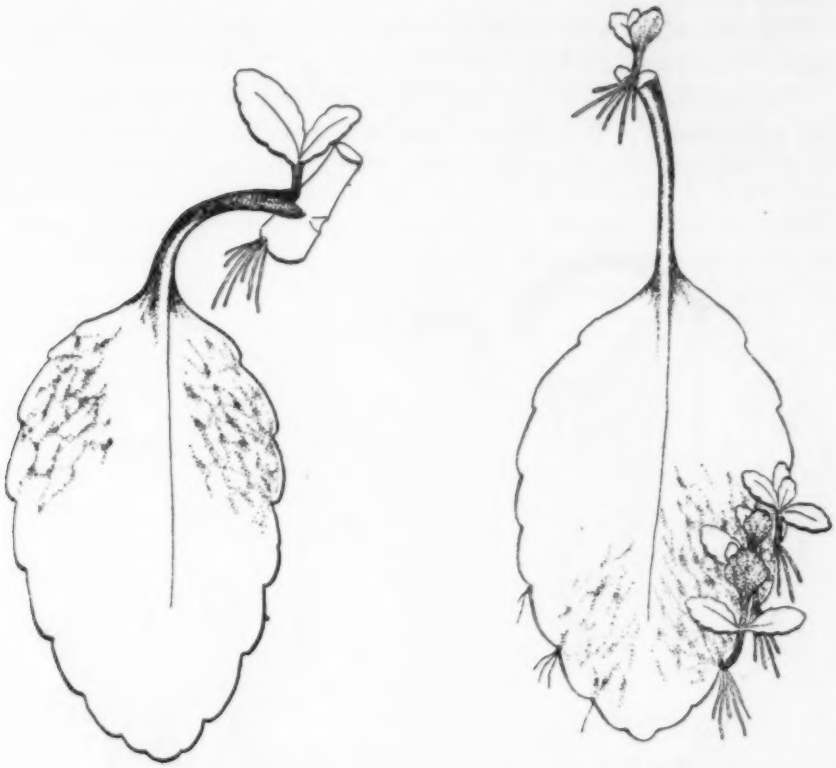

FIG. 2. Two sister leaves suspended entirely in air. The stippling indicates the reddish pigment. In the leaf to the left which is connected with a piece of stem the pigment flows into the axillary shoot of the stem, into the petiole, and is collected in the upper part of the leaf nearest the petiole. In the sister leaf without stem it collects near the place where the new shoots are formed and in the new shoots. Duration of experiment, Feb. 17 to Apr. 5.

newly formed shoots and on its way to these organs. These observations support the assumption that the inhibitory effect of the piece of stem on shoot formation in a leaf is due to the fact that the material available for shoot formation in the leaf flows naturally into the

stem. It also illustrates the principle that when in one part of the leaf growth is very rapid the growth in other parts of the leaf is retarded or inhibited for the reason that the flow of material is towards the rapidly growing organs.

These drawings were made later than those given in a previous paper,[3] which were for this reason less striking.

The inhibitory effect of a small piece of stem on a leaf does not last permanently. It is possible that at first the piece of stem grows in the way described thus inhibiting growth in the leaf, but that later

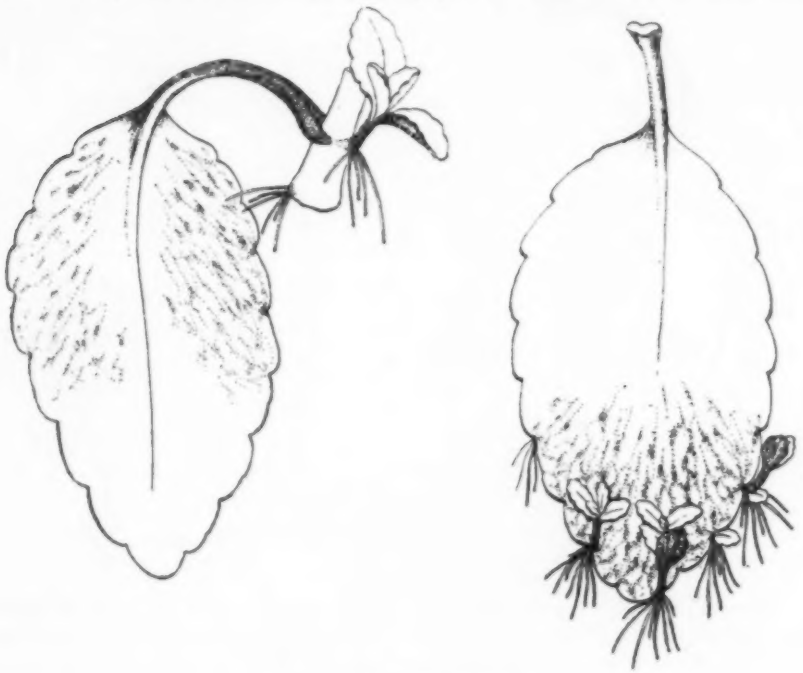

FIG. 3. The same as Fig. 2.

the rate of growth in the piece of stem diminishes, and parallel with this the inhibition of the piece of stem on the growth of roots and shoots in the leaf diminishes also.

[3] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 373.

SUMMARY.

This paper contains the results of a reexamination of a law expressed in a previous paper; namely, that when a piece of stem inhibits the growth of shoots and roots in a leaf connected with it the dry weight of the stem increases and that this gain equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. This has been confirmed and it has been shown that the gain of the stem as a rule even exceeds slightly the mass of shoots and roots the leaf would have produced if it had not been inhibited by the stem. This supports the idea that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available and required for this process naturally flows into the stem.

THE REVERSAL OF THE SIGN OF THE CHARGE OF COLLODION MEMBRANES BY TRIVALENT CATIONS.

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I.

The reversal of the sign of charge of collodion membranes (treated previously with a protein) by hydrogen ions has been discussed in a preceding paper[1] and in this paper the influence of trivalent cations on the reversal will be considered. We shall omit the discussion of the action of tetravalent cations (*e.g.* $ThCl_4$) since their solutions have so high a concentration of hydrogen ions that this alone suffices to bring about a reversal in the sign of charge.

We will prove first that collodion membranes which have previously been treated with a protein give the reversal in the sign of charge in the presence of trivalent cations, while collodion membranes not so treated do not show the reversal. When a solution of $CaCl_2$ of not too high a concentration (*e.g.* below M/8) is separated from pure water by a collodion membrane, which is negatively electrified, the solution shows no attraction for water, while it attracts water powerfully when the membrane is charged positively. The Ca ion acts as if it repelled positively charged water and as if it attracted negatively charged water. To find out whether or not trivalent cations reverse the sign of charge of the membrane we have to add a low concentration of a "neutral" salt with trivalent cation to the solution of $CaCl_2$. Weak solutions of $CeCl_3$ and $LaCl_3$ satisfy this condition. The solutions of $CaCl_2$ are for this purpose made up in M/1,024 solutions of $CeCl_3$ or $LaCl_3$ instead of in distilled water; and the distilled water surrounding the collodion bag is also replaced by

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

M/1,024 $CeCl_3$, so that in regard to $CeCl_3$ or $LaCl_3$ the liquid is the same on both sides of the membrane. Any osmotic effect can therefore only be due to the $CaCl_2$. This allows us to investigate the question of the reversal of the sign of charge of the membrane by the Ce ions. Fig. 1 shows that the $CeCl_3$ can only reverse the sign of charge of the membrane if the latter has previously been treated with gelatin. The lower curve gives the initial rate of diffusion of water (after 20 minutes) into $CaCl_2$ solutions (of different concentra-

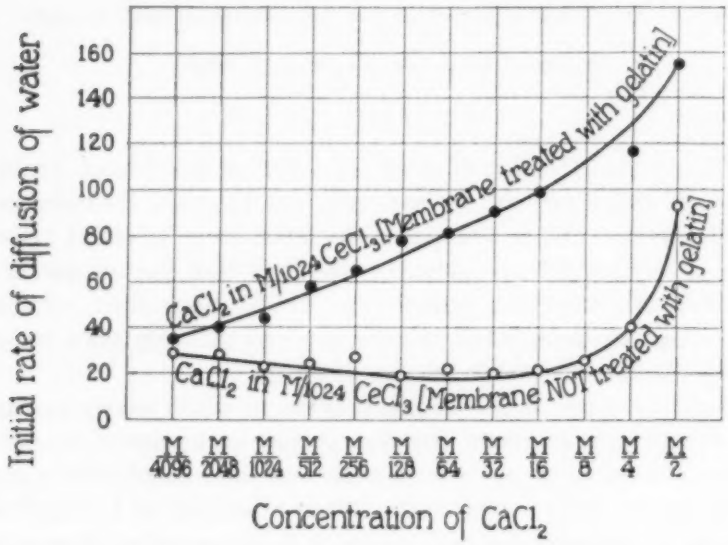

FIG. 1. Showing that $CeCl_3$ renders a collodion membrane positive only when it has been treated with a protein, since $CaCl_2$ below M/16 attracts water only when the membrane is positively and the water negatively charged.

tion) through collodion membranes not treated with gelatin. The membrane is negatively charged since no rise of the level of water in the solution occurs as long as the concentration of $CaCl_2$ is below M/8. The rise of the curve at a higher concentration has a different cause which need not be considered in this connection. The upper curve gives the influence of the same $CaCl_2$ solutions on the rate of diffusion of water from pure water into solution when the membrane has previously received a gelatin treatment. In this case the level of liquid rises in the solution and the more so the higher

the concentration of the $CaCl_2$ solution. The $CeCl_3$ has therefore caused a reversal in the sign of charge of the membrane making the latter positive. This made it possible for the $CaCl_2$ solution to increase the rate of diffusion of water through the membrane into the solution. The pH of the solutions varied between 5.1 (in the lowest concentration of $CaCl_2$) and 5.9 (for the highest concentration); *i.e.*, the solutions were all on the alkaline side of the isoelectric point of gelatin.

Experiments with electrical endosmose confirmed the conclusion that in the presence of $CeCl_3$ the membrane assumes a positive charge when the membrane had previously been treated with gelatin, but that the membrane remains negatively charged when it has not been treated with a protein. In these experiments with electrical endosmose the solutions inside and outside the collodion bag were solutions of $CeCl_3$ of identical concentration.

II.

We have shown in another paper[2] in this number of the *Journal* that in the case of collodion membranes treated with gelatin and rendered positive by acid the rate of diffusion of water from the side of pure water through the membrane to the solution is raised by cations in the order K < Na < Li < divalent cations < trivalent cations. Since the Ce ions render the gelatin film of the collodion membrane positive the influence of different cations on the attraction of water should increase in the same order as when the membrane is rendered positive by acid; and the attraction of water by the solution should be a minimum in the case of K. Fig. 2 shows that this is true. In these experiments different concentrations of the four salts, KCl, NaCl, LiCl, and $CaCl_2$, from M/2,048 to 1M were made in M/1,024 $CeCl_3$. The H_2O surrounding the collodion bags containing the solutions was replaced by M/1,024 $CeCl_3$ to eliminate the osmotic effect of $CeCl_3$ in the experiments. The experiments show that the attraction of water by the four salts follows the order we should expect if Ce caused the membrane to assume a positive charge; *i.e.*, K < Na < Li < Ca. The pH in these experiments varied from

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

5.2 to 6.0, thereby proving that the Ce reversed the sign of the charge of the gelatin film on the inside of the membrane without raising the hydrogen ion concentration to that point where an acid reversal occurs. In Fig. 3 M/1,024 $AlCl_3$ solutions are used for rendering the membrane positive. In this case the pH was about 4.1 in all solutions.

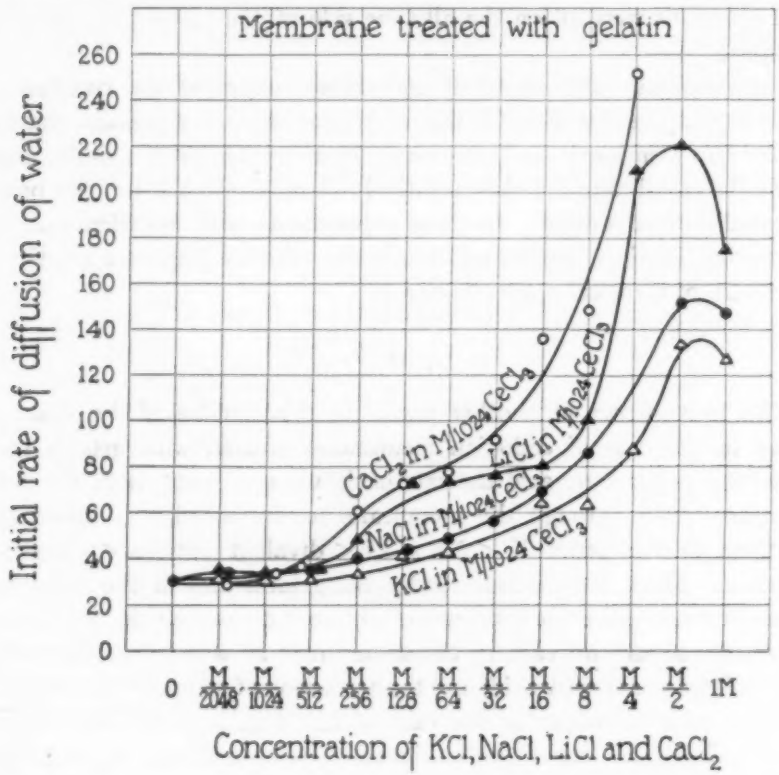

FIG. 2. The relative influence of different cations upon the attraction of water is in the erder Ca > Li > Na > K, proving that the membrane is positively charged in the presence of Ce.

The order of efficiency of cations remains the same as when $CeCl_3$ is used, but the effect is quantitatively larger in Fig. 3 than in Fig. 2.

All these experiments and many others show that trivalent cations cause the membrane previously treated with gelatin to be charged positively on the alkaline side of the isoelectric point of gelatin.

III.

The next question was as to the minimal concentration of a trivalent cation required to render the membrane positive. For this purpose

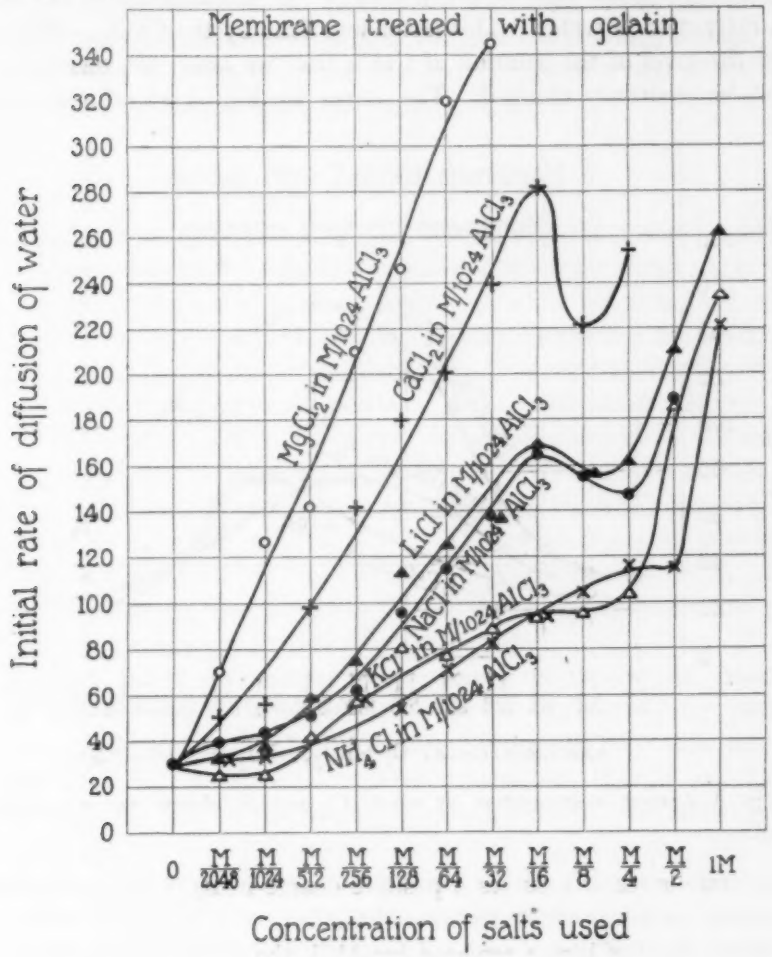

FIG. 3. Showing that Al charges the membrane positively.

various concentrations of $CeCl_3$ were prepared, beginning with M/65,536 $CeCl_3$. To each of these solutions so much $CaCl_2$ was added that the concentration was M/256 in regard to $CaCl_2$. These

solutions were put into collodion bags previously treated with gelatin. The bags were put into beakers containing the same solutions of $CeCl_3$ as that inside the bag but free from $CaCl_2$. Hence the Ce acted only on the sign of the charge of the membrane but not on the attraction of water. This latter was done by the $CaCl_2$. Whenever the level of the solution of $CaCl_2$ rises we know the membrane must be positively charged. The curve in Fig. 4 shows that the

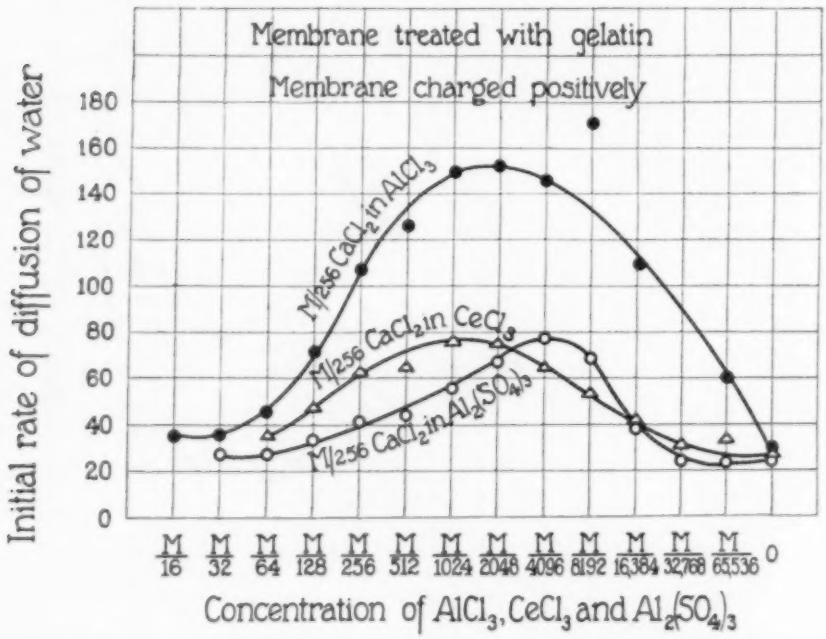

FIG. 4. Lowest concentration at which Ce and Al charge the membrane positively.

membrane already assumes a positive charge in M/32,768 $CeCl_3$ and probably in lower concentrations of $CeCl_3$.

When the $CeCl_3$ was replaced by $AlCl_3$ the membrane assumed a positive charge when the concentration of $AlCl_3$ was less than M/65,536. The pH of this latter solution of $AlCl_3$ was 5.0, so that the reversal was due to the Al ion and not to the hydrogen ion concentration. The fact that Al is more efficient than the Ce (or La) ion may be connected with the fact that the ionic radius of Al is considerably

smaller than that of either Ce or La. The drop in the curves when the concentration of the $CeCl_3$ or $AlCl_3$ solution exceeds M/1,024 is due to the concentration of the anion, as is shown by the fact that the drop is greater in the case of $Al_2(SO_4)_3$ than in the case of $AlCl_3$. This latter phenomenon has been discussed in previous papers. The pH of the $Al_2(SO_4)_3$ solutions almost coincided with that of the $AlCl_3$ solutions of the same concentration.

IV.

It is therefore certain that the trivalent ions bring about a positive electrification in the collodion membrane in contact with water when the membrane has been treated with a protein, but that they cannot reverse the sign of the charge of collodion membranes not so treated. This indicates that the reversal is at least partly due to an action of the trivalent cations on the protein. A chemical combination can only occur between Ce or Al and gelatin on the alkaline side of the isoelectric point where the gelatin is capable of combining with metals. In the experiments described thus far the pH of the $CeCl_3$ solutions was with one exception always > 4.7. The question arises, how will a $CeCl_3$ solution act on the acid side of the isoelectric point of the protein forming the inner lining of the collodion bag where the membrane is already rendered positive by the acid?

M/256 $LaCl_3$ solutions were prepared at different pH, from 7.0 to 2.6, by adding KOH or HNO_3 to the distilled water used for the solutions as required for the pH. These solutions were put into collodion bags and the latter were dipped into H_2O of exactly the same pH as that of the solutions inside the bag. The initial rise of water (in the first 20 minutes) inside the bags was then observed. Similar experiments were made with $CeCl_3$. The collodion membranes had previously been treated with gelatin. Fig. 5 gives the results. It is plain that the curves consist of two distinct parts and that the dividing line seems to lie near the isoelectric point of gelatin. On the alkaline side of the point, *i.e.* for pH 4.7 or above, the initial rate of diffusion is near 140 mm.; and it varies very little with a change of pH. As soon, however, as the pH falls to 4.7 or below, the rate of diffusion rises steeply to 300 and 340 mm. for the two salts.

To understand this result we must discriminate between the effects of the trivalent cations on the reversal of the sign of charge and on the increase of the rate of diffusion of water into the solution, which seems to be due to an increase in the density of charge of a membrane already positive. On the alkaline side of the isoelectric

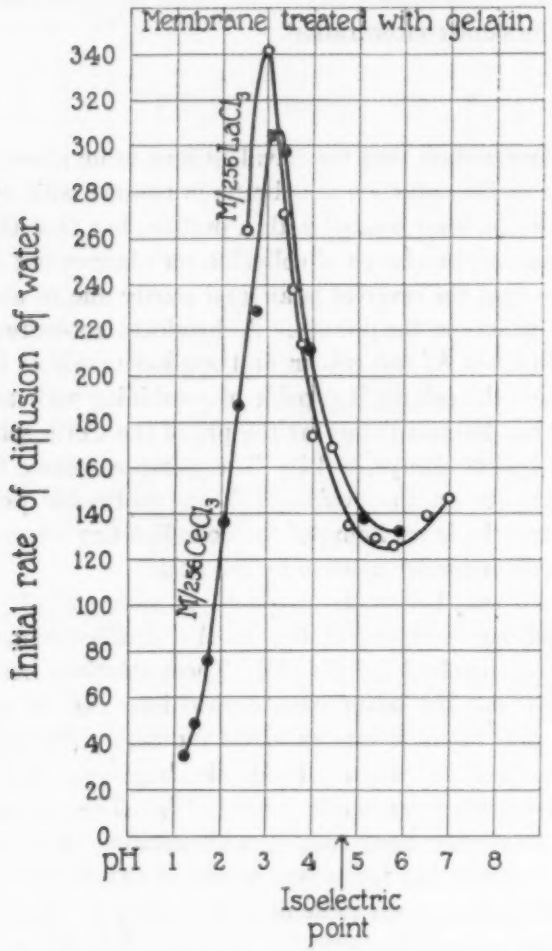

FIG. 5. Although M/256 solutions of La and Ce charge the membrane positively on the alkaline side of the isoelectric point of gelatin, their attraction for water rises very steeply on the acid side of the isoelectric point of gelatin.

point of gelatin the trivalent cation makes the membrane positive by combining with gelatin and forming a salt; *e.g.*, La gelatinate. But, in addition, the salts of trivalent cations attract the water, and the more the higher their concentration until a certain maximum is reached, as shown in Fig. 4. On the acid side from the isoelectric point the protein film cannot react chemically with the La or Ce and the membrane is charged positively through the influence of the

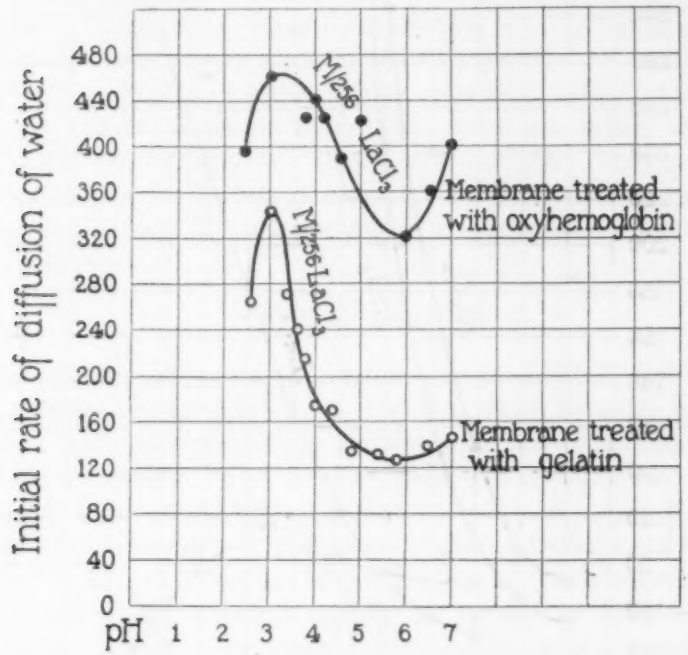

FIG. 6. Showing that the critical point for this steep rise varies with the isoelectric point of the protein with which the membrane has been treated.

acid. In this case the trivalent cation acts apparently by increasing the density of charge of the positive membrane considerably, as was to be expected.

In order to test this idea further, membranes treated with oxyhemoglobin instead of with gelatin were tried. The isoelectric point of oxyhemoglobin is at pH = 6.8. In this case the steep rise in the curve with $LaCl_3$ should be nearer this value than 4.7. The curves

of Fig. 6 show that this is the case. It may be mentioned incidentally that the initial rate of diffusion of water into solutions was greater when the membranes had been treated with oxyhemoglobin than when they had been treated with gelatin. We shall return to this phenomenon in another connection.

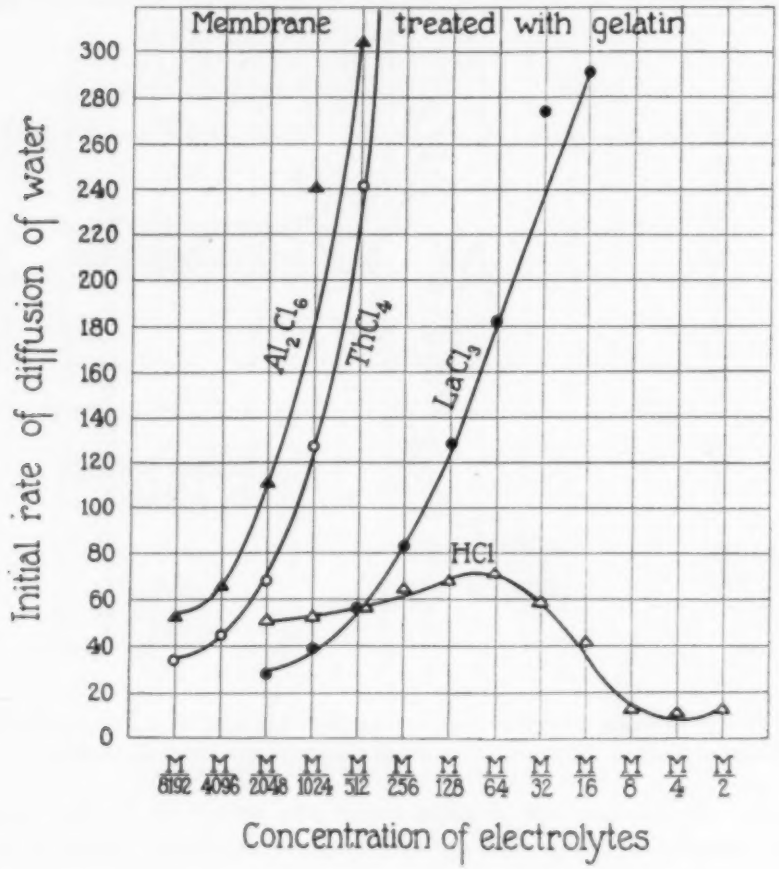

FIG. 7. Showing that the attraction of salts with trivalent cations for water is considerably greater than the attraction of acid (HCl) for water, although acids are very efficient in making the membrane positive.

V.

The experiments represented in Figs. 5 and 6 seem to show that the efficiency of ions in causing the reversal of the sign of charge is as great in the case of hydrogen ions as in the case of trivalent cations; while the influence on the rate of diffusion of water is considerably greater in the case of trivalent cations than in the case of hydrogen ions. Fig. 7 permits a comparison of the attraction of water by solutions of equal concentration of HCl, $LaCl_3$, Al_2Cl_6, and $ThCl_4$ through membranes treated with gelatin. On one side of the membrane was the solution, on the other pure water. If we consider only concentrations up to M/32 this attraction is very slight in the case of HCl as compared with $LaCl_3$ or Al_2Cl_6. (It should be stated that in the experiments with HCl the level of the water was considerably higher at the beginning of the experiment than in the other solutions.)

Theoretical Remarks.

The experiments have shown that a reversal of the sign of charge of a collodion membrane can be brought about by acids and by "neutral" salts with trivalent cation after the inside of the membrane has been in contact for an hour or more with a sufficiently strong solution of a protein whereby apparently a protein film is formed on the inside of the membrane. Collodion membranes not treated are always negatively charged in contact with water, no matter whether or not hydrogen ions and trivalent cations are present in sufficient concentration. Collodion membranes when treated with a protein are also negatively charged when the reaction of the solution is on the alkaline side of the isoelectric point of the protein and when the solution is free from trivalent or tetravalent cations. In this case the protein exists in the form of a metal proteinate dissociating into a positive metal ion, the protein adhering to the inside of the collodion membrane. This might suggest that the membrane becomes negative in contact with water on account of these metal ions dissolving in water, while the solid film of protein anions adhering to the membrane is negatively charged. When the hydrogen ion concentration is raised sufficiently to cause the transformation of the protein

film into a protein-acid salt the reversal in the sign of charge of the membrane would be easily intelligible since the protein-acid salt dissociates into a positive protein cation forming the surface film of the membrane, and an anion which goes into solution.

A difficulty arises, however, if we try to explain the reversal of the sign of charge of the membrane by trivalent cations on the alkaline side of the isoelectric point of the protein. In this case the protein film consists of La or Ce proteinates which are practically insoluble. Practically no dissociation into a negative protein anion and a trivalent cation would be supposed to occur and it seems not possible to state why an insoluble La proteinate should assume a positive charge when in contact with water. As long as this fact is not explained it remains doubtful whether the tentative explanation just given for the acid reversal is correct or complete. It also remains to be explained why the collodion membrane not treated with a protein always assumes a positive charge regardless of the hydrogen ion concentration or the concentration of trivalent cations. It might be argued that the collodion membrane differs from proteins in not being an amphoteric electrolyte and in not being able to combine with trivalent cations. Another possibility must, however, be considered; namely, that the hydrogen ions and trivalent cations influence the surface electrical potential of the solution and that the combined effects of these ions on the surface electrical potential of the membrane and of the liquid determine the phenomena described in our papers.

SUMMARY.

1. Trivalent cations cause a collodion membrane covered with a protein film to be charged positively while they do not produce such an effect on collodion membranes not possessing a protein film. The same had been found for the reversal of the sign of charge of the membrane by acid.

2. This reversal in the sign of charge of the membrane by trivalent cations occurs on the alkaline side of the isoelectric point of the protein used; while the reversal by acid occurs on the acid side of the isoelectric point.

3. The reversal seems to be due to or to be accompanied in both cases by a chemical change in the protein. The chemical change which occurs when the hydrogen ions reverse the sign of charge of the protein film consists in the formation of a protein-acid salt whereby the H ion becomes part of a complex protein cation; while the chemical change which occurs when trivalent cations reverse the sign of charge of the protein film consists in the formation of an insoluble and therefore sparingly or non-ionizable metal proteinate.

IONIC RADIUS AND IONIC EFFICIENCY.

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I. INTRODUCTION.

When pure water is separated from solutions of electrolytes of not too high a concentration (generally below M/4) by a collodion membrane, the initial rate of diffusion of water through the membrane from the side of pure water to that of the solution is influenced in a characteristic way by ions and the following rules have been found to govern this influence.

1. Ions possessing the same sign of electrical charge as the membrane increase and ions with the opposite sign of charge diminish the initial rate of diffusion of water.

2. The relative influence of the oppositely charged ions is not the same for all concentrations. In the lowest concentrations of electrolytes the influence of the ions with the same sign of charge prevails, increasing at first with increasing concentration of the electrolyte until a maximum is reached; a further increase in concentration diminishes the rate of diffusion of water into the solution and the more so the higher the concentration of electrolyte. The turning point varies for different electrolytes but seems to lie for a number of neutral solutions at about M/256 or above.

3. The influence of ions on the initial rate of diffusion of water from the side of pure water through the membrane into the solution increases with their valency and with a second constitutional quantity which in the earlier papers I designated arbitrarily as the radius of the ions.

It will be shown in this paper that the second constitutional quantity seems indeed to be, for monatomic and monovalent ions, the radius of the ion and that the rule connecting the efficiency of ions with

their radius is as follows: *The efficiency of anions increases directly and that of cations inversely with the radius of the ion.* By radius of the ion we mean the distance between the positive nucleus of an ion and its outermost ring or shell of electrons. This term has a definite meaning in the case of monatomic ions.

The method of experimentation is the same as in the preceding publications. The solution of the electrolyte was put into collodion flasks of about 50 cc. content, which had previously been filled over night with a 1 per cent gelatin solution. The gelatin solution was then carefully washed out with warm water. The membrane retained on its inside a very thin film (probably of only one molecule in thickness) of gelatin. This was done to enable us to reverse the sign of charge of the membrane with dilute acid; when the hydrogen ion concentration is below 2×10^{-5} N the membrane is negatively (and the watery phase positively) charged; while when the hydrogen ion concentration is above this value the membrane assumes a positive and the water a negative charge. The membranes used in the experiments to be described had therefore all been treated with gelatin.

The collodion flask was closed with a rubber stopper, through which a glass tube with a bore of about 2 mm. in diameter was pushed inside the flask. The latter was filled with the solution of electrolyte and suspended in a beaker with distilled water having the same hydrogen ion concentration as the solution of electrolyte. The glass tube served as a manometer to indicate the rate at which water diffused from pure water into the solution through the collodion membrane. The temperature was kept constant at 24°C. We shall treat the influence of ions on negatively and positively charged membranes separately.

II. Negatively Charged Membranes.

When an electrical double layer is formed at the boundary of a collodion membrane (previously treated with gelatin as described) the membrane assumes a negative and the watery phase a positive charge as long as the hydrogen ion concentration is below 2×10^{-5} N and no trivalent or tetravalent cations are present. If the hydrogen ion concentration exceeds 2×10^{-5} N and becomes 10^{-4} N the sign of charge on the two layers is reversed. This was proved by experi-

ments on electrical endosmose.[1] We will first describe experiments in which the membrane is negatively and the watery phase positively charged.

We have shown in a previous paper that anions accelerate and cations depress the rate of diffusion of water through negatively charged membranes.[2] Fig. 1 shows the relative influence of the anions of three potassium salts, KCl, KBr, and KI on the rate of diffusion of water through collodion membranes. The ordinates

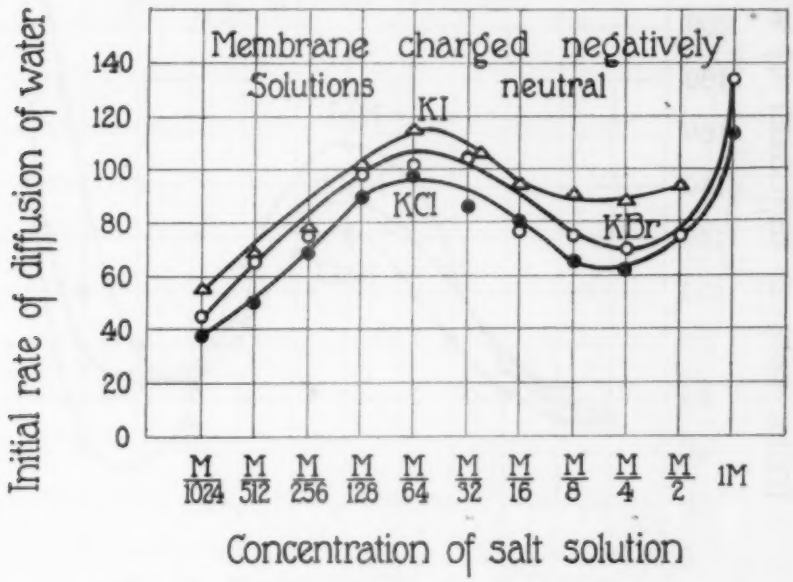

FIG. 1. The rate of diffusion of water through a negatively charged membrane (from the side of pure water to solution) and the density of the charge of the latter are increased by monovalent, monatomic anions in the order I > Br > Cl. Solutions almost neutral (pH about 6.0).

indicate the rise of liquid in the manometer in 20 minutes; the abscissæ indicate the concentration of the solution. The salt solutions as well as the pure water separated from the solution by the collodion membrane were almost neutral, having a hydrogen ion concentration of about 10^{-6} N. It is obvious that the accelerating influence of

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.
[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

anions on the rate of diffusion of water from pure water into the solution through a negatively charged membrane increases in the order I > Br > Cl, the accelerating influence of the I ion being the greatest.

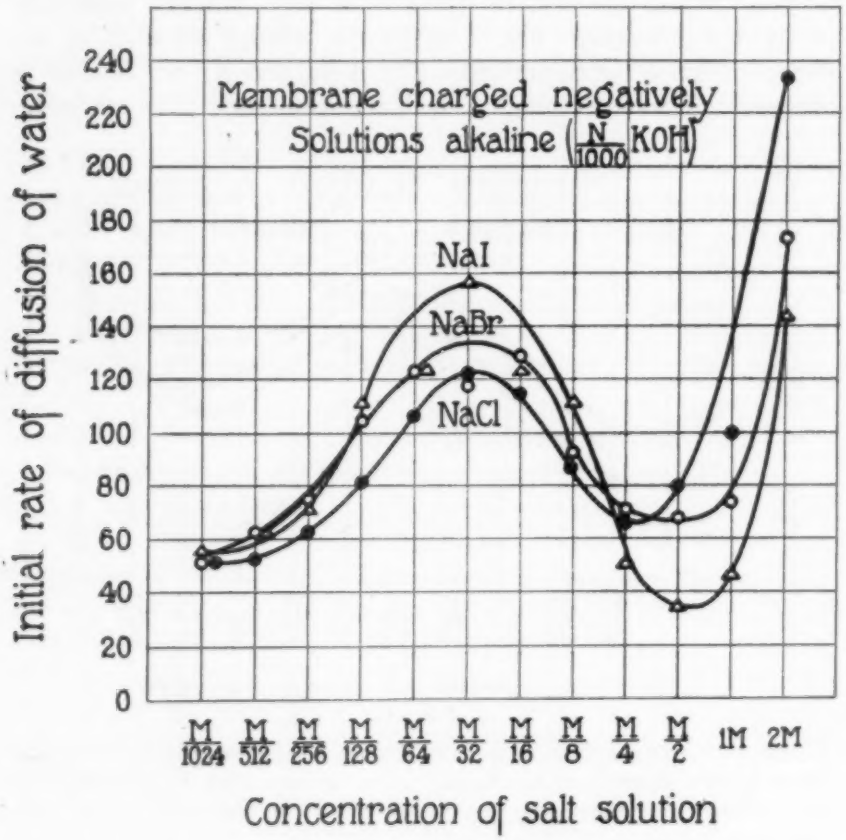

FIG. 2. The same as Fig. 1 except that the solutions and water are alkaline (pH about 11.0).

The same is true when the solution is more alkaline. Thus in Fig. 2 NaCl, NaBr, and NaI were dissolved in a N/1,000 solution of KOH instead of in pure water and the outside solution was N/1,000 KOH. The sign of charge of the membrane remained negative, however, and therefore the relative efficiency of the three anions

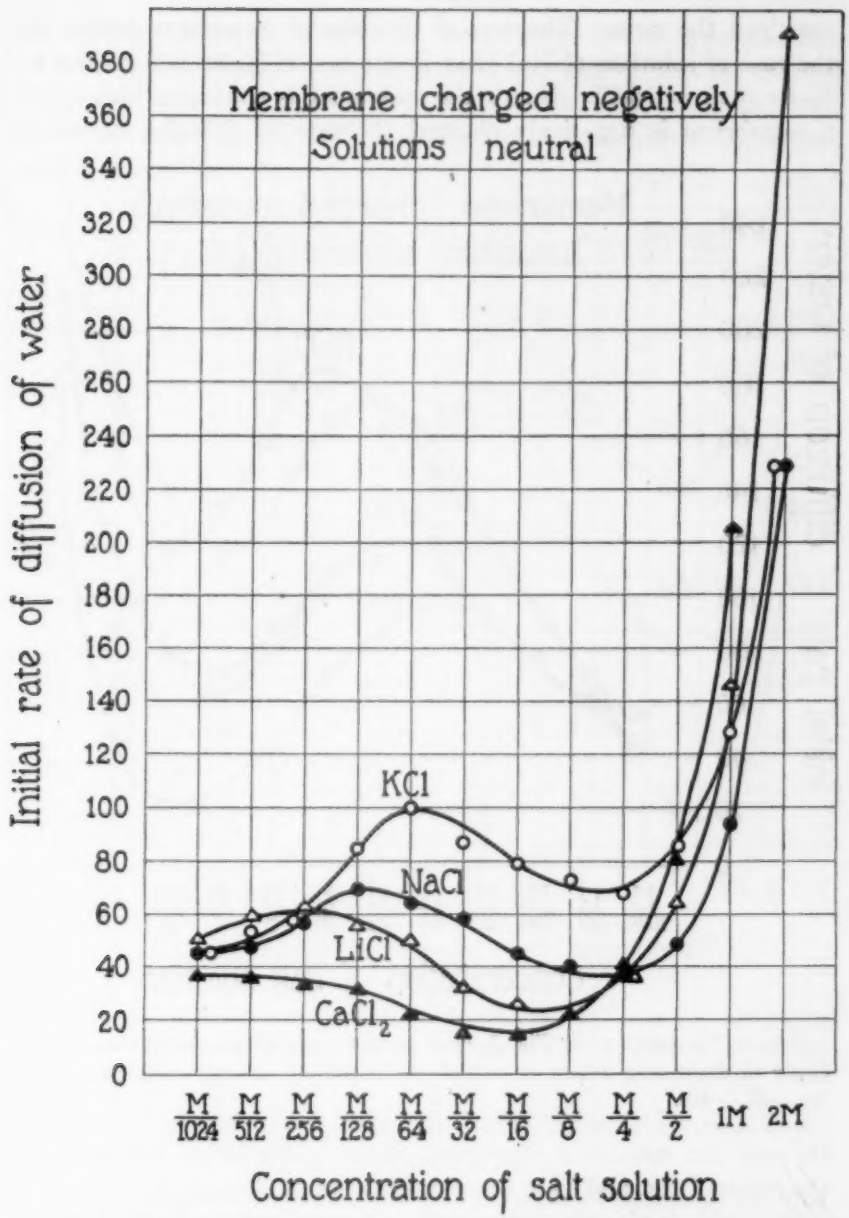

FIG. 3. The rate of diffusion of water through a negatively charged membrane and the density of charge of the latter are depressed by monovalent, monatomic cations in the order Li > Na > K. Solutions neutral (pH about 6.0).

remained the same. The rate of diffusion of water was greater in the case of solutions of NaI than in the case of NaBr, and greater in NaBr than in NaCl. Hence the statement is confirmed that when a membrane is negatively charged the rate of diffusion of water

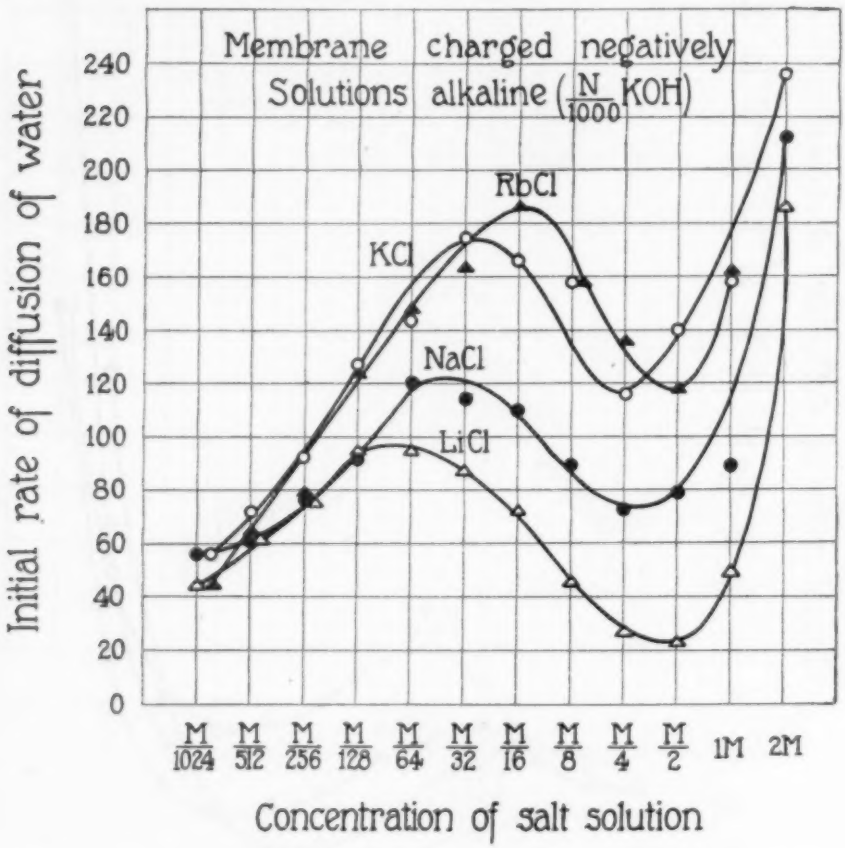

FIG. 4. The same as in Fig. 3 when solutions are alkaline (pH about 11.0). Order of depressing action of cations on negatively charged membrane Li > Na > K > Rb.

through the membrane from pure water into solution increases for the anions I, Br, and Cl in the order I > Br > Cl.

Since this is also the order of the magnitude of the ionic radius of the three anions, I having the greatest radius, we can say that the

accelerating effect of monatomic, monovalent anions upon the rate of diffusion of positively charged water through negatively charged membranes from the side of pure water to the side of the salt solution increases directly with the order of magnitude of the radius of the anion.

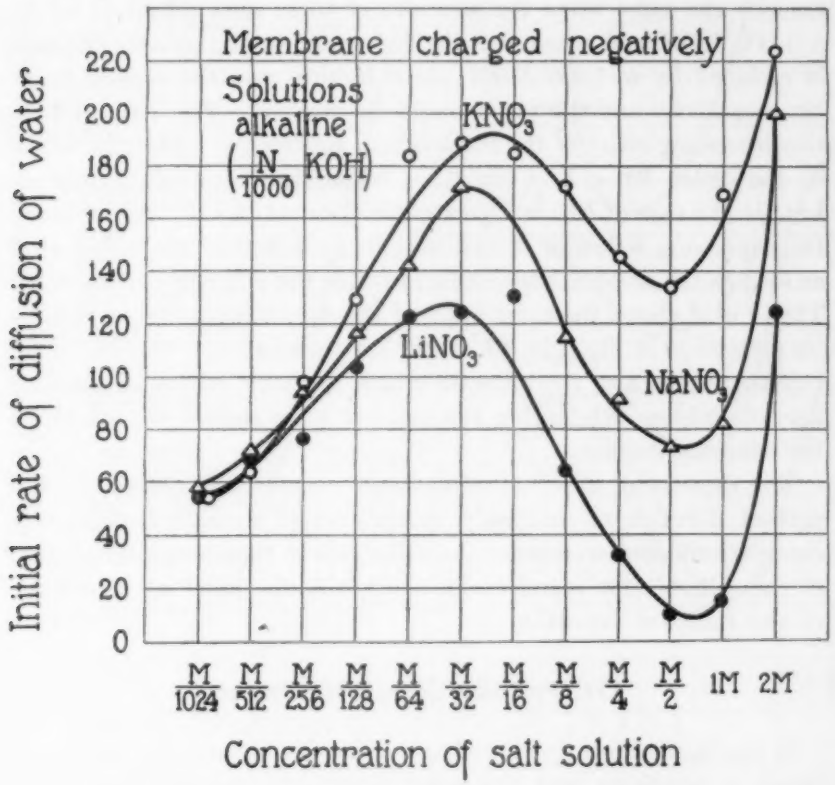

FIG. 5. The depressing order of cations upon negatively charged membranes is the same for NO_3 as for Cl (Fig. 4). Solutions alkaline, order of depressing effect Li > Na > K.

The same relative order of efficiency for the three anions was found also when they were in combination with Li.

While the anions increase the rate of diffusion of positively charged water through a negatively charged membrane cations diminish it and the question arises in what order the depressing effect of the

monovalent cations increases. Fig. 3 shows that in the case of monovalent cations the depressing effect is least in the case of K, and increases in the following order, K < Na < Li.

In these experiments the solutions were almost neutral (hydrogen ion concentration about 10^{-6} N). The order of efficiency of the cations remains the same when the solutions of these salts are made up in N/1,000 KOH and when the distilled water in the outside solution is replaced by N/1,000 KOH; since in this case the membrane is also negatively and the water positively charged. Fig. 4 shows that the depressing effect of the monovalent, monatomic cations increases in the order Rb < K < Na < Li, where the depressing effect is least in the case of Rb, and greatest in the case of Li. This order of the depressing influence of cations is independent of the anion used as long as the anion is always the same for the different cations used. Thus Fig. 5 shows the same order of the depressing action of cations for nitrates as in Fig. 4 in the case of the chlorides.

Since this is also the order in which the ionic radius of the four ions diminishes (Rb having the greatest ionic radius) we can make the following statement.

The depressing effect of monatomic, monovalent cations on the rate of diffusion of positively charged water through a negatively charged collodion membrane from the side of pure water to the side of the salt solution increases inversely with the order of magnitude of the radius of the cation.

III. Positively Charged Membranes.

It has been shown in preceding publications that when the membrane is positively and the water negatively charged the cations increase and the anions diminish the rate of diffusion of water from the side of pure water through the membrane into the solution. In order to make a membrane, previously treated with gelatin, positively and the watery phase of the double layer negatively charged it is necessary to give the water and the solutions a hydrogen ion concentration of about 10^{-4} N or above. We made the solution acid by dissolving the salts in 10^{-3} N HNO_3 and the distilled water outside the collodion bag was also replaced by 10^{-3} N HNO_3. The hydrogen

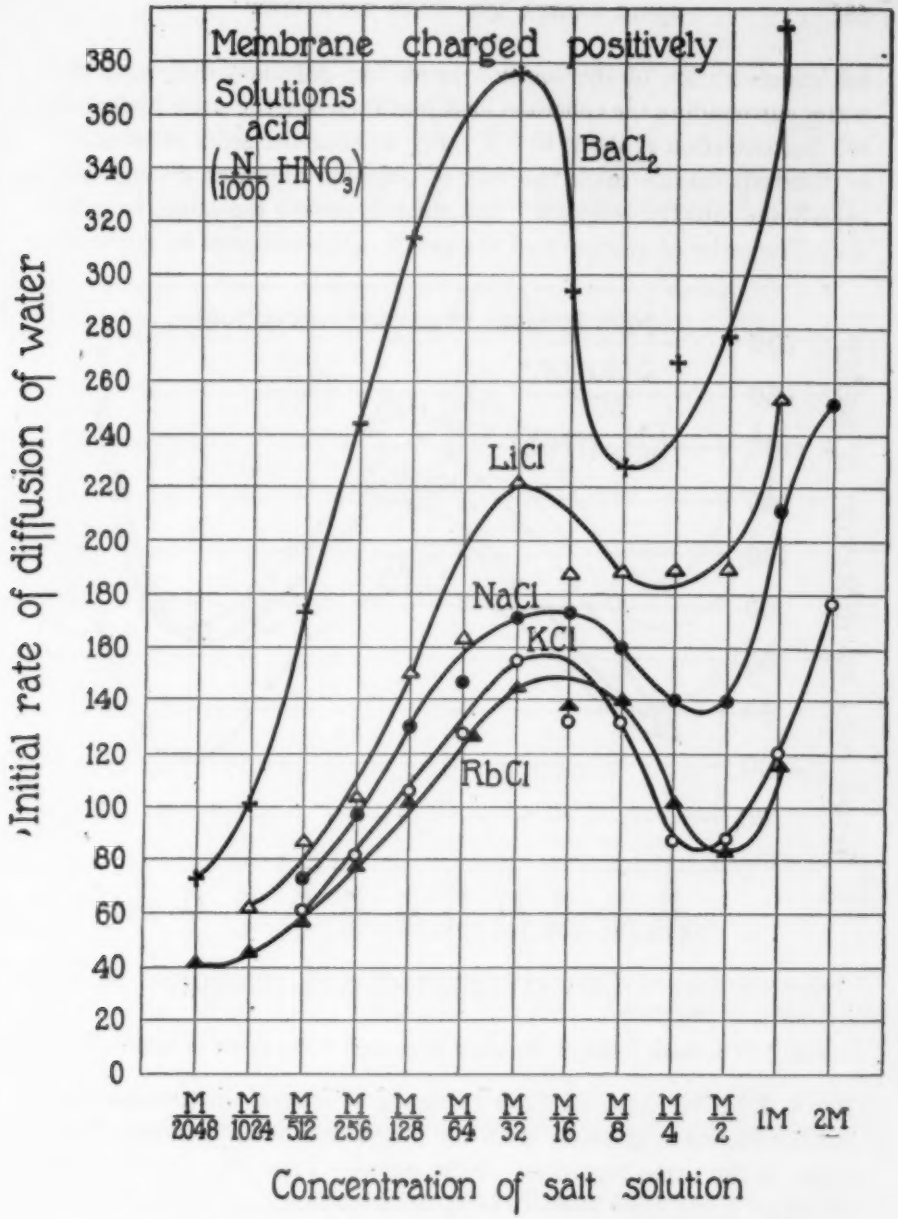

FIG. 6. The rate of diffusion of water through a positively charged membrane and the density of charge of the membrane are increased by the monovalent, monatomic cations in the order Li > Na > K > Rb. Solutions acid (pH about 3.0).

ion concentration of the solution inside the collodion flask and the water surrounding the collodion flask had therefore the same hydrogen ion concentration of about 10^{-3} N. Fig. 6 shows the order of influence of different cations upon the rate of diffusion of water through the membrane into the solution. The anion is always the same; namely, Cl. The order of efficiency of the cation series increases in the order

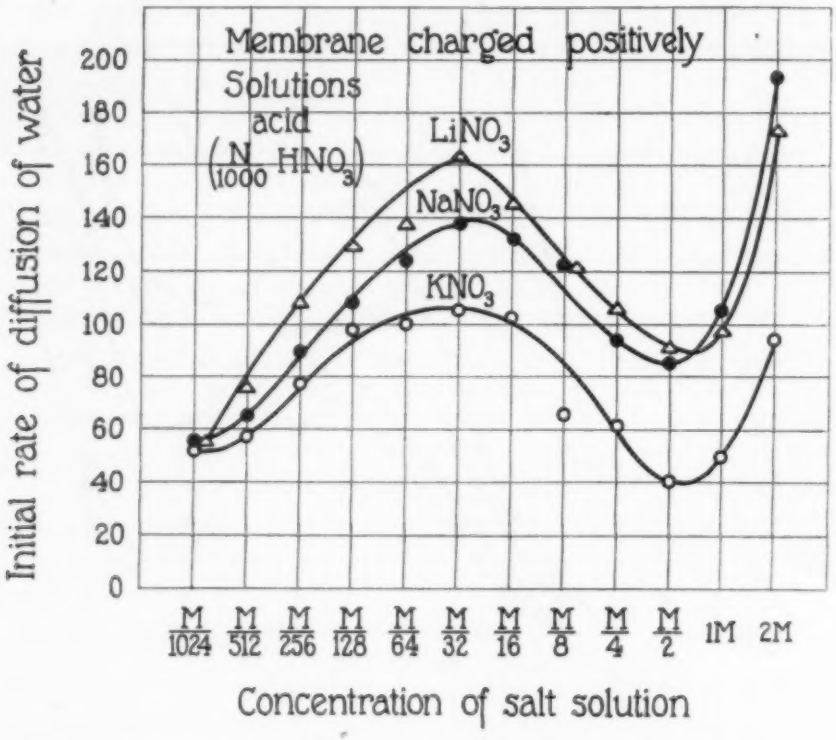

FIG. 7. The same order of efficiency of cations if the anion is NO_3.

Rb < K < Na < Li; *i.e.*, the efficiency of monovalent, monatomic cations increases inversely with the magnitude of their radius. This order is the same regardless of the anion. Thus Fig. 7 shows that the order is the same when Cl is replaced by NO_3.

The anion depresses the rate of diffusion of negatively charged water through the membrane into the solution when the membrane is positively charged. Fig. 8 shows that this depressing effect of

different potassium salts increases in the order I > Br > Cl, where the depressing effect of I is greatest.

This order is the same regardless of the nature of the cation. Thus Fig. 9 shows the same order of depressing effect of the anion on the rate of diffusion of water through positively charged membranes in the case of barium salts.

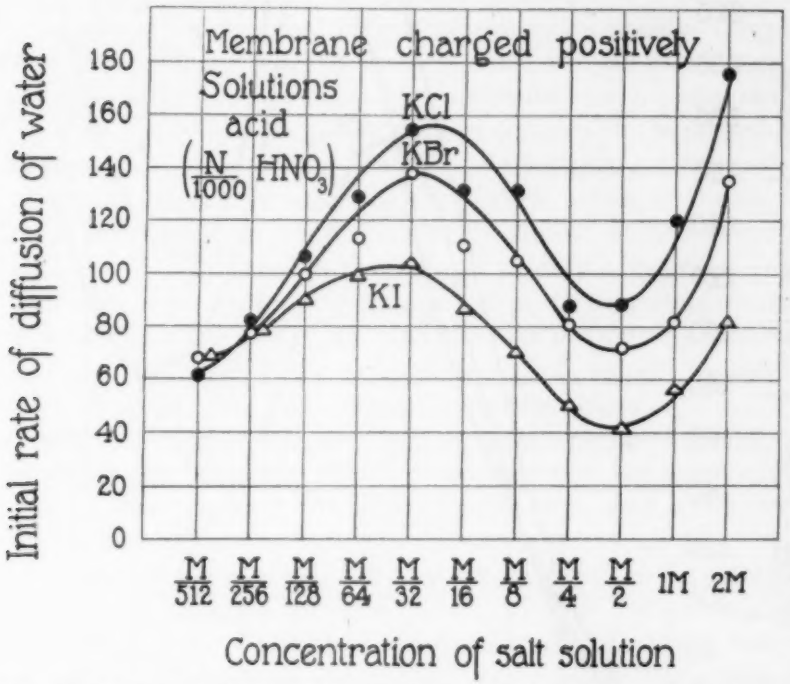

FIG. 8. The rate of diffusion of water through positively charged membranes (pH about 3.0) and the density of charge of membrane are depressed by anions in the order I > Br > Cl.

If we combine the results of the experiments on positively and negatively charged membranes we can express them in the following form.

The accelerating and depressing effect of monatomic, monovalent ions on the rate of diffusion of water through a collodion membrane from the side of pure water to the side of the solution increases for anions directly and for cations inversely with the magnitude of their radius.

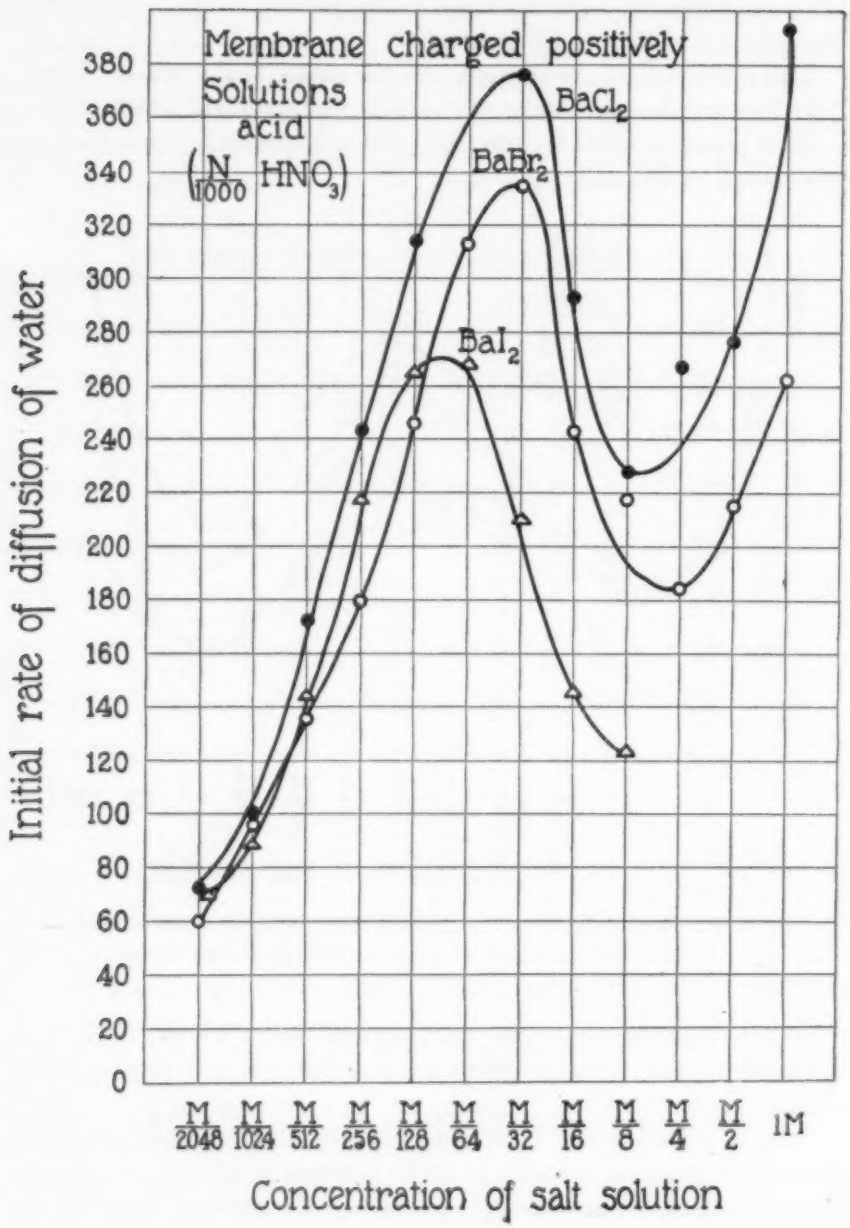

FIG. 9. The same as Fig. 8 for a different cation.

IV. Theoretical Considerations.

Kossel[3] has suggested that for chemical reactions of atoms we may replace the atoms by simplified models consisting only of the charges of the positive nuclei in the center and the ring of valency electrons. The distance between the positive nucleus and the outermost ring or shell of electrons we call the radius of the atom or of the ion. While a monatomic, monovalent cation has one excess positive charge in its nucleus a monatomic, monovalent anion has one excess negative charge in its periphery. Although these positive and negative charges are alike, the relative electrostatic action of ions with the same charge must vary with the radius. Cations act through the excess positive charge on the nucleus, and the electrostatic action of the nucleus must become the greater the smaller its radius; *i.e.*, the nearer the positive nucleus can get to the body on which it is supposed to act. This explains why the accelerating as well as the depressing efficiency of a cation on the rate of diffusion of water through the membrane increases *inversely* with the radius of the cation; namely, in the order Rb < K < Na < Li, where Rb has the minimal effect.

When the nucleus has more than one excess charge the accelerating as well as the depressing efficiency increases with the valency, since *ceteris paribus* the electrostatic action of a cation must increase with the number of excess charges of its nucleus.

We understand also why the accelerating as well as the depressing efficiency of an anion increases *directly* with its radius, since the electrostatic effect of a monovalent anion on an outside body is determined by its excess electron and hence will be the greater the greater the distance between the valency electron and its positive nucleus.

It is also obvious why the efficiency of an anion should increase with its valency; *i.e.*, with the number of valency electrons it contains in excess of its nuclear charges.

We can only speak of ionic radius in the case of monatomic ions like K or Cl; when, however, an ion consists of more than one atom, *e.g.* NO_3, CNS, we have more than one positive nucleus and each nucleus has its own rings or shells of electrons. The term ionic

[3] Kossel, W., *Ann. Physik*, 1916, xlix, 229.

radius loses its meaning in this case unless we substitute for such polyatomic ions a monatomic model with one nucleus, one ring of valency electrons and a radius calculated in such a way as to render the electrostatic effect of the monatomic model equal to that of the polyatomic ion which it is supposed to represent.

The rule at which we arrived, namely that the efficiency of the anions increases directly with their ionic radius while the efficiency of the cations increases inversely with their ionic radius, is probably of general applicability in physical chemistry as well as in physiology, wherever the efficiency of ions depends on their electrostatic action.

There are numerous observations in the physiological literature which indicate an inversion of the order of efficiency of monovalent cations when the reaction of the solution changes from basic to acid. It is possible that these facts will find their explanation on the basis of our rule; namely, that on one side of the neutral (or in certain cases the isoelectric) point the effect observed is increased by the cation and inhibited by the anion, while on the other side it is increased by the anion and inhibited by the cation. Unfortunately the facts given are often too incomplete to test this idea and the experiments are generally done with such high concentrations of electrolytes that it is doubtful whether they can be used for any conclusions concerning the specific effects of ions.

SUMMARY.

1. It has been shown in preceding papers that when we separate solutions of electrolytes from pure water by collodion membranes the ions with the same sign of charge as the membrane increase while the ions with the opposite sign of charge diminish the rate of diffusion of water from the side of pure water to the side of solution; and that the accelerating and depressing effects of these ions on the rate of diffusion of water increase with their valency.

2. It is shown in this paper that aside from the valency a second quantity of the ion plays a rôle in this effect, namely the radius, which in a monatomic ion means the distance between the central positive nucleus and the outermost ring or shell of electrons of the ion. In monatomic, monovalent anions the radius increases in the

order Cl< Br< I (being largest in I), while for monatomic, monovalent cations it increases in the order Li< Na< K< Rb (being largest in Rb).

3. It is shown that the accelerating as well as the depressing effect of the anions mentioned increases directly with the order of magnitude of their radius and that the efficiency is greatest in the case of I which has the largest radius; while the accelerating as well as the depressing effect of cations increases inversely with the order of magnitude of their radius, Li with the smallest radius having the greatest efficiency.

4. This is intelligible on the assumption that the action of the ions is electrostatic in character, in the case of cations due to the electrostatic effect of the excess charge of their positive nucleus, and in the case of anions due to the excess charge of their captured electron. The electrostatic effect of the positive nucleus of a cation on the membrane (or any other body) will be the greater the smaller the ionic radius of the cation; and the electrostatic effect of an excess electron will be the greater the further its distance from its own positive nucleus.

5. It is suggested that this rule may possibly include polyatomic, monovalent ions (*e.g.* NO_3, CNS, etc.) when we replace these polyatomic ions by monatomic models in which the radius is calculated in such a way as to give the model the same electrostatic effect which the polyatomic ion possesses.

6. These conclusions are in harmony with the fact that the efficiency of ions increases also with their valency.

7. It is suggested that these rules concerning the influence of the ionic radius can possibly be demonstrated in other phenomena, depending on the electrostatic effect of ions.

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